Mohamed Lotfy Taha Elsaie

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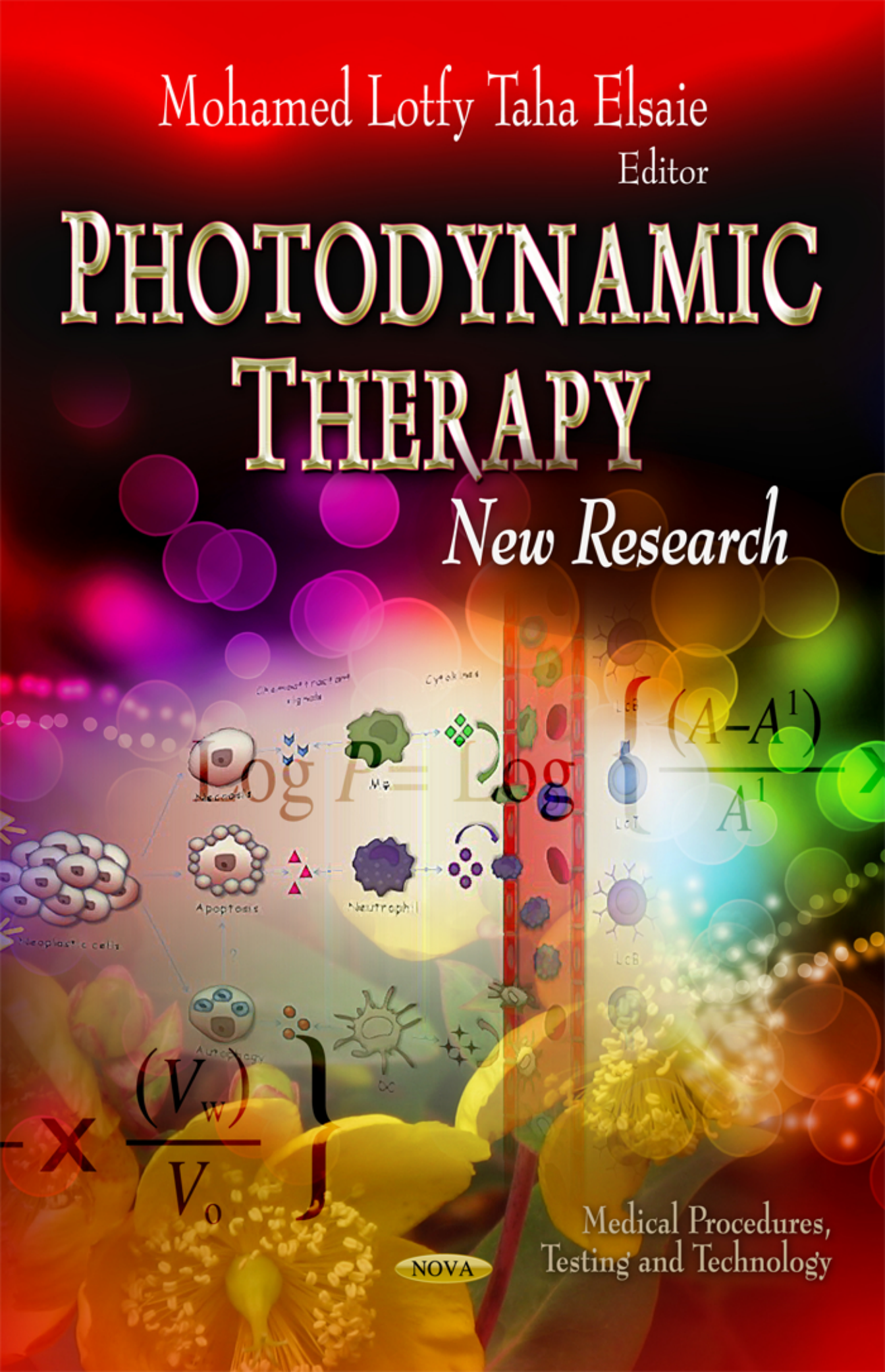

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MEDICAL PROCEDURES, TESTING AND TECHNOLOGY

PHOTODYNAMIC THERAPY

NEW RESEARCH

MOHAMED LOTFY TAHA ELSAIE
EDITOR

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This book is dedicated to:

My family and friends who have been there for me in every instance of my life and were always inspirational,

My Parents Prof. Dr. Lotfy Elsaie and Prof. Dr. Laila Meky,

My brothers Dr. Ahmed Elsaie and Engineer Mostafa Elsaie and to the rest of my dear family,

A very special dedication to my beloved wife Dr. Eman Ahmed Youssef for always being there for me.

I love you all

Contents

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Mohamed L. Elsaie, MD, MBA

Introduction

Photodynamic therapy (PDT) is currently clinically employed to treat several malignant and nonmalignant diseases. With approvals for various applications by health agencies in Europe, the US, Canada, and Japan, PDT represents the method of choice for treatment of age-related macular degeneration and is appreciated as minimally invasive therapeutic procedure to treat skin, oesophageal, head and neck, lung, and bladder cancers with high cure rates, nearly no side effects, and excellent cosmetic outcome. Current basic research and clinical studies will help to further integrate PDT as a mainstream procedure for cancer treatment. Motivated by the outstanding success of PDT in management of various human diseases, new and very promising applications of the combination of a photosensitizing agent and illumination with light were identified and explored by the PDT research community. So, for example, photodynamic inactivation (PDI) of microorganisms has the potential to overcome the severe threat of increasing microbial resistance against conventional antibiotics. Also, photodynamic procedures are applicable as photoinsecticides or to disinfect sensitive surfaces.

The concept of PDT began with studies by Oscar Raab in 1900 on the effects of light and dyes on Paramecia. The modern era of PDT began with studies by Lipson and Schwartz at the Mayo Clinic in 1960, who observed that injection of crude preparations of hematoporphyrin led to fluorescence of neoplastic lesions that could be visualized during surgery. Since then, considerable work has been done on how the process works, how to maximize efficacy using animal models, and how to best treat human tumors. These pre-clinical and clinical studies recently resulted in the approval of the first photosensitizing drug (Photofrin®) for the treatment of selected tumors. first photosensitizer to gain regulatory approval for clinical PDT was Photofrin, but, due to its several disadvantages, particularly prolonged patient photosensitivity, second and third generation photosensitizers were investigated.

In 1902, Georges Dreyer in Copenhagen examined the effect of light on bacteria. Besides bacteria and animal skin, he also sensitized living human skin to demonstrate the phototoxic effect. In 1903, Dreyer started his first experiments in patients with lupus vulgaris by intra- and subcutaneous injection of a sterile erythrosine solution and illumination after four to eight hours. Within 24 hours, a severe phlegmonous reaction resulted which resolved leaving prominent scar formation. The patients suffered from severe pain during irradiation and Dreyer therefore, terminated his experiments. In contrast, von Tappeiner and Jesionek reported on good results using topical application of eosin or other dyes. In 1905, they

extended their trial on patients with superficial skin cancer and observed efficacy with repetitive photodynamic therapy (PDT) using topically applied 0.1% to 5% eosin dye.

Today, it is known that PDT requires the simultaneous presence of a photosensitizer, light, and oxygen inside the diseased tissue. The photosensitizer accumulates in the target cells and absorbs light of a certain wavelength. The energy is transferred to oxygen and highly reactive oxygen species, mainly singlet oxygen, are generated. Following an appropriate light dose, the reactive oxygen species (ROS) directly lead to cell and tissue damage by inducing necrosis and apoptosis or indirectly stimulate inflammatory cell mediators.

The second generation photosensitizers are generally single substances and not necessarily porphyrins, and have improved selectivity and activity. The third generation photosensitizers have an additional targeting mechanism, for example, by covalent attachment to monoclonal antibodies. These photosensitizers have been designed to improve the selectivity of uptake and to take advantage of the greater depth of penetration of light of longer wavelengths than that used to activate Photofrin®. Clinical trials are being conducted with these photosensitizers at many centers around the world in an effort to provide information for drug approval

Like many of the new treatments that are currently being researched, photodynamic therapy has considerable potential. But as the results of recent trials emerge, just as there's certain to be good news, there will also be more questions raised. So while we're fully supportive of efforts to improve and research PDT, clinicians, researchers and scientists feel that there's a need for a sense of perspective about the treatment.

PDT uses a simple concept, but the multiple parameters involved in it make it complex, and more research is required to determine the exact parameters optimal for the treatment of each disease. This would include factors concerning the photosensitizing agents, such as mode of delivery and duration of application, and parameters relating to light, such as wavelength, duration and intensity.

Photodynamic therapy(PDT) is used in the management of neoplastic and nonmalignant diseases. Preclinilal studies indicate that PDT can induce local microenvironmental changes to facilitate formation of antitumor adaptive immune response; PDT also triggers rapid infiltration of the diverse populations of immune cells to the tumor site, induce complement cascade and produce cytokines/chemokines, eventually the anti-tumour adaptive immunity will be promoted. PDT could produce the cancer vaccine. In recent years, many preclinical studies have indicated that the therapeutic outcome of PDT can be improved by combination with immunotherapy.

The book covers novel areas of mechanistic and innovative translational approaches. Moreover, it presents an overview of the important medical applications of PDT, including approved treatments, clinical trials, and investigated therapies for cancer and non-malignant diseases.

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Chapter 1

Contrasting Facets of Nanoparticles-Based Phototherapy: Photo-Damage and Photo-Regeneration[1]

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Abstract

The present review describes two different applications of the phototherapy. The first one referred as Photodynamic Therapy (PDT) is widely used for pathologically proliferated tissues, including cancer. PDT is based on the combined action of three parameters: photosensitizer, light and oxygen, resulting in the production of toxic reactive oxygen species. To improve the selectivity of photosensitizers for pathological tissues the liposomal photosensitizers formulations have been proposed favoring tumor targeting. The comprehension of the mechanisms that govern the drug release processes of photosensitizers in liposomal nanostructures and as such their pharmacokinetics behavior is essential and is addressed in the present review.

Another facet of nanoparticle-based photoinduced therapy is related to tissue regeneration. Complex mechanisms of photoinduced wound healing and repair will be considered, including the role of different cell types, growth factors and antimicrobial action.

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In conclusion, PDT offers two contrasting strategies either to eradicate pathological tissues or to stimulate tissue repair. In fine, consideration of all parameters involved in this kind of phototherapy is required to obtain a desired effect and optimize clinical protocols.

Introduction

Photodynamic Therapy (PDT) is regarded as an exposure therapy used in clinical settings. This treatment strategy is based on the combined action of a molecule called the photosensitizer (PS), light and molecular oxygen. PDT involves the PS administration followed by local light illumination of targeted tissues at the appropriate wavelength to activate the PS. The molecule can further react with oxygen to produce damaging reactive oxygen species (ROS).

This photoinduced oxidative stress is the basis of damaging effect of PDT resulting in destruction of pathological tissues. PDT is applied for cancer treatment, specifically for small localized tumors accessible to the visible light irradiation (bladder, skin, head and neck, esophageal…). Tumoricidal effect of PDT is triggered by a direct damage of malignant cells producing cell death by necrosis and/or apoptosis. PDT also affects tumor vasculature, causing the shutdown of vessels with subsequent depriving of the tumor with oxygen and nutrients. Finally, PDT can immunostimulate or immunosupress the immune system. A serious limitation of photodynamic therapy (PDT) is the absence of specific cancer targeting, resulting in an excessive tissue destruction, which can provoke life threatening situations. To partially overcome these constraints, incorporation of the active compound into liposomal nanoparticles can be proposed. Embedding of active drugs in liposomes favours passive targeting of tumors through Enhanced Permeability Retention (EPR) effect. Liposomal formulations of second-generation photosensitizers enable a more selective and more rapid accumulation of drugs in the tumors, with a faster clearance, together with a better efficiency. The possible difficulties consist in specific photosensitizer behaviour in bulk lipid milieu. Therefore, a comprehension of redistribution patterns of PSs from liposomes of different motifs to biological structures *in vitro* is essential. Kinetic parameters will directly influence spatial intratumoral distribution of PSs and provide indications to the photosensitizer transport from the vessels towards extravascular structures. The visualization of these processes in real time *in vivo* is a crucial point. The model that is particularly well adapted to study vasculature effects of exposure therapy treatments is the chick chorioallantoïc membrane (CAM). Advantages of this model for PDT effects are described in the present review. *In fine*, consideration of all above mentioned parameters that will rend possible an optimization of clinical protocols is the core of present review.

Another facet of nanoparticle-based photoinduced therapy is related to the improvement of tissues regeneration, mostly referred here as wound healing or repair. The grounds of photo-induced tissues repair are complex and not completely elucidated, the main mechanisms to consider are photoinduced immunomodulation, microorganisms' inactivation and growth factors stimulating production. In function of the photosensitizer, light dose and the type of tissue wound healing process can be altered, stimulated or unchanged.

Thus, beneficial and damaging effects of phototherapy will be discussed in the present review.

1. The Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) is a therapeutic strategy for the treatment of localized tumors accessible to light illumination. It consists of the systemic or local administration of a molecule called "photosensitizer" (PS) which accumulates selectively in malignant tissues due to physiological alterations in the pathological environment like *e.g* leaky vasculature, abnormal enzymatic activity, pH variations or reduced lymphatic drainage. The local irradiation of the diseased area activates the PS combined with light and molecular oxygen to lead to the generation of cytotoxic reactive oxygen species (ROS) [1].

Photochemical reactions of type 1 and type 2 constitute the origin of the efficacy of the PDT treatment. In the Type 1 reaction, the PS reacts directly with a substrate (cell membrane or a molecule) to form radicals which can further react with molecular oxygen to produce ROS. Alternatively, in the Type 2 reaction, the triplet PS can transfer its energy directly to the molecular oxygen to form excited state singlet oxygen or perform an electron transfer to molecular oxygen and generate superoxide anion radical. Both Type 1 and Type 2 reactions can occur simultaneously and the ratio between them depends on the PS used and the concentration in substrates and oxygen [2]. Due to the high reactivity, short diffusion distance (0.01 µm - 0.02 µm) and short half-life of singlet oxygen (40 ns) and hydroxyl radicals, only molecules and structures that are proximal to the production site (*i.e* PS localization site) are directly affected by PDT [3, 4]. The tumoricidal effect of PDT is triggered by the direct damage of malignant cells and/or indirect vascular damage accompanied by an immune response. The contribution of each type of damage depends on different parameters: the type of the PS and its localization in the tumor, the rate of vascularity and macrophage content in the tumor [5].

Photosensitive agents and light have been used for medical purposes for a long time. The first health agency approval for PDT was obtained in 1993 in Canada for the prophylactic treatment of bladder cancer with Photofrin® [1]. Regulatory approvals for the clinical use of PSs and PDT light applicators now exist in many countries around the world though the total number of approved clinical indications is still limited. Potential clinical applications of PDT are diversified [6]. Dermatological, ophthalmic and cardiovascular diseases are considered but here we shall focuse on oncological diseases. Different types of cancer were tested for the treatment by PDT and some of them were finally approved : head and neck cancers, urological and gynecological cancers [7].

1.1. Dosimetry

The optimization of the applied dosimetry in PDT treatment is still a very important research topic passing through the study of the major parameters of interest: the photosensitizer administration (route, conditions, formulation), the time of the administration and irradiation and the irradiation procedure (delivered light intensity) [8].

1.1.1. Drug Light Interval (DLI)

PS distribution in the tumor can be modulated by the drug light interval (DLI) which corresponds to the time that separates the PS administration from the illumination [9]. Indeed,

compartmental targeting of the tumor microvasculature or parenchyma is closely related to the PS distribution, governed by the pharmacokinetic and cell/tissue binding properties of the PS.

Vascular damage are observed when irradiation is performed with short DLI which corresponds to vascular localization of the PS [10-12]. On the contrary, for long DLI, direct tumor cell destruction is obtained when the PS content is high within the tumor cells [13, 14]. The mechanism responsible of the tumor cell destruction depends on the intracellular localization of the PS. Photosensitizers can be located in the mitochondria, the endoplasmic reticulum, the plasmic membrane or lysosomes and PDT treatment can trigger the cell death either by apoptosis or necrosis [15-20].

1.1.2. Light Delivery

The choice of the light source, irradiation wavelength, light dose and intensity are primordial for a successful PDT treatment. The light source must exhibit suitable spectral characteristics that coincide with the maximum absorption wavelength range of the PS applied in order to generate enough ROS to produce a cytotoxic effect [2]. A critical parameter for consideration in discussing the efficacy of PDT is the depth of light penetration through tissues which is dependent on several processes including reflection, scattering, transmission, absorption or a combination of these. The 620 to 800 nm wavelength range is often called the "therapeutic window" where light penetration is maximal (3-8 mm for 630-800 nm) [21]. Photodynamic dose treatment is described as a fluence (J/m^2) and a fluence rate (W/m^2). It has been nicely demonstrated with biological outcomes, such as skin photosensitivity, clonogenic assays, tumor regrowth assay and tumor cure rates, that PSs have lower efficacy when high optical fluence rates are used [22, 23].

1.1.3. Oxygenation

Oxygen is absolutely necessary for an effective PDT treatment. Numerous studies performed *in vitro* and *in vivo* demonstrated that lack of oxygen diminishes the PDT efficiency [24-26]. Several techniques have been proposed to deal with tissue oxygen depletion during PDT such as fractionating light irradiation or reducing the fluence rate [26-28]. These techniques are to promote tissue oxygen re-perfusion to compensate for the oxygen depletion caused by the photochemical reactions. Particularly, the modulation of the irradiance was shown to be directly related to the preservation of oxygen conditions during the treatment correlated with a better mTHPC-PDT outcome [22, 29, 30]. As an example, Coutier *et al* observed that tumors treated with fluence rates of 5 and 30 mW/cm^2 exhibited significantly longer tumor quadrupling times than those treated at 160 and 90 mW/cm^2 and the tumor regrowth profile correlated with the pO_2 values monitored during irradiation. Improved tumor destruction can be expected by reducing the rate and the extent of oxygen depletion using low fluence rates.

1.1.4. Photosensitizers

The prerequisites for an ideal sensitizer include chemical purity, selectivity for malignant cells, chemical and physical stability, low dark but strong photocytotoxicity, activation at wavelenghts with optimal tissue penetration and rapid clearance from the body without inducing significant skin photosensitivity [31]. Those properties are resumed by optimal

Absorption, Distribution, Metabolism and Excretion (ADME). Moreover, photosensitizers must have a high **singlet** oxygen quantum yield, not induce mutagenic or carcinogenic effects and be inexpensive and commercially available [32]. The first porphyrins used in PDT did not respect these guidelines, were inhomogeneous, poorly defined mixtures of different hematoporphyrins extracted from blood and therefore conducted to higher skin photosensitization and a lack of selectivity [33]. Most of the current photosensitizers have porphyrin-related structures, including hematoporphyrin derivatives, phtalocyanines, chlorins and bacteriochlorins which all exhibit different photochemical and photophysical properties in terms of mechanisms of action and light activation [31]. O'Connor *et al* made an exhaustive article presenting different types of PSs and their characteristics [32]. A PS can be administrated exogenously, or endogenously and produces photosensitive metabolites (*e.g* protoporphyrin IX from 5-aminolevulinic acid). Majority of photosensitizers are hydrophobic and form aggregates in biological environment making difficult intravenous administration and reducing PDT efficiency. The monomer form of PS is generally most photophysically active compared to aggregates [9]. To overcome this problem of aggregation, PSs are generally either formulated in various colloidal drug delivery systems such as liposomes, micelles and biodegradable nanoparticles or conjugated with hydrophilic polymers [34, 35]. Plenty of newly designed PS have been patented and tested in preclinical studies but only few of them gained marketing authorization, mainly due to the difficulty to reach all the requisites cited above.

1.2. Liposomal Formulations of Photosensitizers

As stated above, the majority of novel photosensitizer molecules are of hydrophobic nature. Although this feature is required to penetrate cellular walls in order to accumulate in cells [36] and to be able to be transported by lipoproteins, it induces immediate aggregation upon injection into aqueous media, thus diminishing the photosensitizer efficacy [37, 38] generally due to the decrease of singlet oxygen formation yield [39, 40]. Therefore, water-soluble drug delivery systems are needed to overcome the problem of photosensitizer aggregation and to maintain the high efficacy of the photodynamic treatment. Additionally, the delivery systems should be preferably designed to provide targeting capabilities, improving the pharmacokinetic properties of the drug formulation, while remaining biodegradable and non-toxic.

1.2.1. Description and Classification

To date, a number of drug delivery systems has been designed, including oil-in-water emulsions, polymer conjugates, micelles, dendrimers, polymer nanoparticles and metal nanoparticle carriers [41, 42], among the most widely studied being the liposomal delivery systems [43]. In this review we will focus on liposomes as delivery agents.

Liposomes are defined as ideally spherical self-closed phospholipid vesicles consisting of one or several bilayers, first discovered by A. Bangham and co-workers almost 50 years ago [44]. Due to characteristic bilayer structure, encircling a certain aqueous volume, liposomes may encapsulate both hydrophobic and hydrophilic drugs [45, 46]. The size of liposomes ranges from about 20 nm to several microns, structurally represented by concentric lipid bilayers of nanometer-scale thickness (*e.g.*, ~ 4 nm for dipalmitoylphosphatidylcholine (DPPC) [47].

Amphiphilic lipids comprising the lipid bilayer consist of hydrophilic polar headgroup and hydrophobic hydrocarbon chains, which structure promotes the spontaneous aggregation of such molecules and the self-formation of bilayer in water [48] under certain circumstances. Depending on the number of lipid bilayers, liposomes are structurally classified into multilamellar and unilamellar vesicles [49]. Unilamellar vesicles have a single lipid bilayer, sub-divided into small unilamellar vesicles (diameter 20-100 nm), large unilamellar vesicles (diameter 100-500 nm) and giant unilamellar vesicles (diameter > 1 micron) [50]. Multilamellar vesicles possess several bilayers, and are of more than 500 nm diameter, reaching up to 10 microns [50]. Liposomes are generally produced by either extrusion through a polycarbonate membrane, sonication, reverse-phase evaporation, or injection method (see [51] for review). The main components of liposomes are generally synthetic (DPPC, dipalmitoylphosphatidylglycerol, dimiristoylphosphatidylcholine, distearoyl phosphatidyl choline) or natural (soy bean, egg lecithin) phospholipids, which provides a basis for biocompatibility.

1.2.2. Liposomal Inclusion of Photosensitizers

1.2.2.1. Photochemical and Photophysical Properties

As summarized by Lang *et al* [38], there are 3 main effects of liposomes on photosensitizers: monomerization of aggregated hydrophobic drugs as a result of localization in the lipid bilayer, a significant increase in the local photosensitizer concentration and the viscosity effect, thus structuring the microenvironment of the drug. Monomerization of hydrophobic sensitizers represents a well-studied effect, shown for a variety of drugs such as hematoporphyrin [36], benzoporphyrin derivative monoacid ring A [52], phthalocyanines [52], and chlorins [53]. The increase in the local concentration may actually lead to altered photophysical properties of photosensitizer. For instance, in Foslip® commercial liposomal formulation, the local concentration of mTHPC reaches 0.1 mM (at only 1 μM in solution) resulting in a phenomenon of Photoinduced Quenching [54]. Another consequence could be the effect inverse to monomerization, that is, aggregation, as was shown for porphyrins [55].

Incorporation of photosensitizers into liposomes changes the photophysical properties of the drugs, such as fluorescence and absorbance characteristics (usually red-shift of the maxima) [56, 57], changed fluorescence quantum yield [38, 53, 58], excited states lifetimes and the yield of singlet oxygen generation, which are sensitive to the microenvironment. An excellent review is available on the photophysical properties of porphyrin sensitizers in liposomal delivery systems [38]. It should be noted that the effect of significant local concentration may also influence the singlet oxygen quantum yield (decrease in case of aggregation), thus directly affecting the treatment efficiency of the formulation [59]. The changes in fluorescent properties of the photosensitizers provide a tool for the quantifying the interactions of the drug with liposomes and serum proteins, as was explored, *e.g.*, in [60, 61], for the interaction of deuteroporphyrin and aluminium phtalocyanine with membrane models and lipoproteins. Polarization of photosensitizer fluorescence in liposomes due to the increased microviscosity in liposomes provides another tool for studying the drug localization in vesicles as well as serving as a phase transition-sensitive probe [38].

However, not only liposomes change the characteristics of photosensitizers upon incorporation, but also the drug itself may change the physico-chemical properties of the formulation. It was recently shown on a clinically approved Foscan® sensitizer (active substance

– mTHPC) that the inclusion of high loads of mTHPC into DPPC-based liposomes significantly shifts phase transition temperature of the liposomes [62]. In case of Foslip® formulation described there, a decrease of more than 5 °C was registered. At the same time, no increase of the liposome size was noted. While the phase transition characterizes the active content release property, this effect implies the complex interdependence of drug and liposomal carrier properties, which need to be verified while studying the newly-developed lipid-based drug formulation. In a different study of mTHPC-loaded liposomes it was shown that at the maximal drug loads the localization of the drug in liposomes may actually change [53]. Indeed, instead of being localized fully in the lipid bilayer, a part of mTHPC was residing in the polymer shell of Fospeg® liposomes, thus also changing the release of the drug from the formulation.

1.2.2.2. Photosensitizer Release

The liposomes being designed as drug delivery systems, the knowledge of the characteristics of the drug release is of utter importance for the successful application of the formulation, and the controlled drug release remains the holy grail of the liposome biotechnology. The extents of drug entrapment and retention as well as the factors influencing them are important considerations in the design of liposome-mediated drug delivery systems. While hydrophilic drugs are retained in the aqueous core of the liposomes, and are subject to diffusion through the lipid bilayer [63, 64], hydrophobic ones retained in the lipid bilayer have minimal entrapment and retention problems due to high lipid-water partition coefficients. The incorporation of cholesterol into the lipid bilayer membrane is a common method to enhance the stability of liposomes, reduce the permeability of the membranes to water-soluble molecules and increase the fluidity or microviscosity of the bilayer [65, 66]. A comprehensive review of the drug release mechanisms from liposomes is available elsewhere [63]. These mechanisms include, among others, diffusion-controlled release, dissolution/degradation release, release by ion exchange, osmosis, external influence (heating or irradiation). It should be noted that the permeability of the lipid bilayer, directly related to drug release properties, is affected by liposome size, pH of the environment and drug properties [63].

1.2.2.2.1. Methods of Investigation

Apart from conventional methods of drug release estimation, such as radiolabeled molecules (*e.g.*, [67]), ultracentrifugation or size-exclusion chromatography, liposome-entrapped photosensitizers offer a distinct method to control the release of the entrapped drug from liposomes. A novel approach to analyze drug transfer to large oil-in-water emulsion droplets by flow cytometry was suggested by Petersen *et al* [68]. Although inapplicable to drug release to nanosized blood components, it estimated the characteristic release times of mTHPC to cell-sized acceptors using flow cytometry technique and registering mTHPC fluorescence. In another example, energy transfer between lipid bilayer-incorporated fluorescent marker and entrapped chlorin (mTHPC) and mTHPC fluorescence anisotropy were applied to estimate the drug release from DPPC/DPPG liposomes [69]. As a peculiar to mTHPC characteristic, the phenomenon of drug photoinduced fluorescence quenching was discovered in mTHPC liposomal formulations with high drug loads [54]. This effect consisted in the formation of small percentage of photoproducts after low-intensity laser irradiation, which, in conditions of efficient homogenous energy transfer between mTHPC molecules in lipid bilayer, quenched the fluorescence of the whole population of mTHPC in a liposome. It

was recently used to study the mTHPC release from Foslip® and Fospeg® liposomal formulations to blood serum proteins, investigating the effect of the temperature and acceptors on the characteristics of drug release [53].

1.2.2.2.2. Processes Implicating in the PS Redistribution

It is generally considered that the photosensitizer redistribution from liposomes may proceed through the aqueous phase or due to collision of drug-loaded liposomes with plasma proteins [70]. In the former case, the rate of the sensitizer transfer is determined by the rate constant of the dye desorption from the lipid bilayer and is independent of the concentration of acceptor structures. In the latter case, the photosensitizer molecules migrate due to collisions between liposomes and plasma proteins. Its rate constant is determined by the frequency of collisions and, as a consequence, by serum concentration. Sometimes the combined mechanism is discussed, as it is applicable to mTHPC [67].

At the same time, the drug release from the liposomes is not the only source of photosensitizer loss from the vesicles. It was reported that direct transfer of phospolipid material to serum lipoproteins may take place [71], mainly as a loss of phospholipids to HDL [72], which is mediated by phospholipids transfer protein [73, 74]. This results in the destruction of liposomes and consequent release of the content. In this case it shall be noted that the lipoprotein association pattern of the drug being released gradually from the disintegrating liposomes may vary from that of the injected liposome-free drug undergoing the stages of desaggregation [75]. Noteworthy, association of the photosensitizer released from the liposomes with LDL particles may increase the uptake of the drug by tumor cells by LDL-mediated endocytosis. Also, the uptake of intact photosensitizer-loaded liposomes by cells in a process of clathrin-mediated endocytosis [76] may change the subcellular localization of the drug, thus changing subcellular cytotoxic events in the course of PDT treatment.

Apart from liposome disintegration, liposomes injected into the bloodstream associate quickly with plasma proteins in a process called opsonization [77] and endocytosed by the cells of mononuclear phagocyte system (MPS) [78], rapidly decreasing the concentration of liposomes in blood circulation. These two effects are responsible for the extremely short half-life of the conventional liposomal formulations in blood, in the range of minutes [79]. Although the rapid uptake is preferred for the treatment of MPS-localized infections [80], the removal of liposomes from circulation is the major disadvantage for other drug delivery purposes including PDT treatment. This may be considered as a drawback compared with the use of liposome-free drugs.

1.2.2.2.3. Improvement of Pharmacokinetic Properties

One of the main methods of prolonging the liposome circulation is the coating the liposome surface with biocompatible PEG polymer (typically 4-6 mol%), forming a layer that prevents rapid opsonization and protein adsorption of the liposome surface [81, 82], increasing the circulation time from minutes to hours. It should be noted that the attachment of PEG to the surface of a liposome does not fully prevent liposome uptake by the reticuloendothelial system but only reduces the uptake rate [83]. One of the most significant advantages of such sterically stabilized liposomes is the nonsaturable, log-linear pharmacokinetics [43]. Sterically stabilized liposomes likely resist uptake by the high-

affinity, low-capacity reticuloendothelial system macrophages, resulting in increased circulation lifetimes [84]. Additionally, it prevents liposomes aggregation during the storage and administration [85]. The drawback of such approach is the reduced ability of PEGylated liposomes to interact with target membranes [51].

This is critical since the singlet oxygen generated by the irradiated photosensitizer shows an extremely short migration radius. For instance, Gijsens *et al* demonstrated that sterically stabilized liposomes containing hydrophilic photosensitiser aluminium phthalocyanine tetrasulphonate did not display any *in vitro* photocytotoxic activity on malignant cells, while the free compound did [86]. Ichikawa *et al* noted that tumor accumulation of benzoporphyrin derivative monoacid ring A at 3 hours after its injection with PEG-liposomes in Meth A-sarcoma bearing mice was significantly higher than the one observed after injection with nonmodified liposomes.

However, significant tumor growth suppression after PDT was only observed for conventional but not for PEGylated formulation [87].

Due to the fact that they exploit the natural distribution pattern (passive diffusion and phagocytosis processes), liposomes, oil dispersions, biodegradable polymeric particles and hydrophilic polymer-PS conjugates are considered as passive targeting systems [34]. Several studies have shown that the selective accumulation in target tissues such as tumors or neovasculature of these targeting carriers is due to the phenomenon known as “Enhanced Permeability and Retention effect” (EPR). Indeed, the tumoral microenvironment presents a vascular defective architecture which increases the tumoral permeability for various vascular factors [88]. The description of the EPR effect and influencing factors are described in the following part of these review.

2. Photoinduced Passive Targeting

Similar to normal tissue, tumor growth depends on a functional vascular network for the delivery of oxygen and nutrients and removal of metabolic wastes. Tumor targeting with PDT can be divided into two different approaches: a passive targeting and an active targeting. Passive photodynamic targeting refers to an approach based on the accumulation of the PS in the targeted compartment as a result of physicochemical factors of drug carriers, such as material composition, size and surface properties (*e.g* electric charge), and by pathophysiological and pharmacological factors such as tumor microenvironment as well as EPR effect [5, 89, 90].

On the opposite, active photodynamic targeting relies on photosensitizer structural modification or a targeted drug delivery system so that the compound can be selectively bound to and retained in the targeted compartment (cellular/vascular) based on molecular recognition. In the present review, we will focuse on the different ways of passive targeting of the vascular (neovasculature) or of the cellular (parenchyma) compartment of the tumor.

2.1. Specific Light Delivery

Selectivity of PDT mainly results from the fact that illumination itself limits the area that is being damaged and includes tumor cells, tumor stroma (including vasculature and fibroblasts) and tumor infiltrating cells of the immune system. Selective tumor targeting can be achieved with PDT by using specific light delivery provided by recent developments in various laser light sources and fiber optic delivery devices [91]. Indeed, the local irradiation of the malignant tissues permit to prevent photoinduced damages to surrounding healthy tissues and target selectively the tumor.

2.2. DLI Modulation: Compartmental Targeting

As cited previously, modulation of DLIs directly influences the local distribution of the PS in the tumor. Short DLIs are used to obtain a vascular localization of the PS in the tumor whereas long DLI permit to have PS in neoplastic cells. Zhou *et al* noticed the importance of the choice of the DLI to obtain a good treatment efficacy together with a maximal protection of normal tissues [92]. Several teams had applied this concept of compartmental targeting to enhance photodynamic efficacy [93-98]. Many subsequent investigations commented on the impact of DLI on the photodynamic effect, with significant improvement of efficiency with shorter DLIs, corresponding to high plasma levels of the drug [99-101]. The single targeting of the cellular compartment or the vascular compartment never concluded in a complete tumor cure. As a consequence, the use of two PS administrations was proposed, each one at different time before irradiation, in order to obtain high drug levels in both cellular and vascular compartments of the tumor. In general, this protocol did not significantly improve the results. Veenhuizen *et al* investigated the impact of a double injection of mTHPC 1-3h and 48h before irradiation but the response of the tumor was better at short DLI even if mTHPC level in the tumor was maximal at long DLI [12]. Identical observations were made by the same team when both injections were separated by 72 hours, despite the fact that the total drug dose was doubled [10]. Using a so-called vascular photosensitizer, Dolmans *et al* [11] demonstrated that a double injection of a pyropheophorbide derivative at 15 minutes and 4 hours before irradiation induced a significant tumor growth delay compared to a single drug dose at any of those times. For them, this improvement was related to a more homogeneous staining of both endothelial and perivascular structures following a double injection. A more recent article presented a very important improvement of PDT efficacy by using the same protocol of fractionation of the injection keeping the same total administrated dose of photosensitizer. Based on previously established intratumoral distribution of mTHPC [102], Garrier *et al* treated mice bearing tumors with a mTHPC fractionated injection at 24 hours and 3 hours before illumination (30 mW/cm², 10 J/cm²) in order to target both tumoral compartments [103]. The fractionated injection protocol, based of the use of two DLIs, yielded a 100% tumor cure correlated to a massive apoptosis of neoplastic cells and destruction of the neovasculature. This study demonstrated the importance of the compartmental targeting which critically influences PDT efficiency.

2.3. Pharmacokinetics: Stability and Bioavailability of Liposomes

2.3.1. Enhanced Permeability Retention Effect (EPR)

Investigations of Matsumura and Maeda firstly described that the majority of solid tumors presented a defective architecture of blood vessels which increases the permeability for various factors [104-113] called the "Enhanced Permeability Retention" (EPR) [113]. The EPR effect is based on this unique anatomical-pathophysiological nature of tumor blood vessels which facilitates transport of macromolecules into tumor tissues. EPR effect was not only observed for proteins but also with drug-polymer conjugates, micelles and liposomes and has thus now become the "gold standard" in anticancer strategies using macromolecular agents [88].

Any liposomal formulation needs to balance the liposomal stability in the circulation with drug availability/release once it arrives at the target tissue. Actually, liposomal technology is still unable to selectively release drugs in the target tissue and different factors, described below, can all affect the liposomal stability and the release of therapeutic agents [35, 83].

2.3.2. Factors Influencing EPR Effect

2.3.2.1. Size, Composition and Charge

The size of liposomes used in a drug delivery system should be large enough to prevent their rapid leakage into blood capillaries but small enough to escape capture by fixed macrophages that are lodged in high quantity in the reticuloendothelial system (RES) [114]. The size of the sinusoid in the spleen and fenestra of the Kupffer cells in the liver varies from 150 to 200 nm [115] and the size of gap junction between endothelial cells of the leaky tumor vasculature may vary from 100 to 600 nm [116]. Consequently, liposomes need to have a size up to 100 nm to reach tumor tissues by passing through these two particular vascular structures [114]. It was observed that an increase of the size of liposomes triggered a more rapid uptake by the reticuloendothelial system (RES) [83, 117, 118]. The dependency of size on liposomal clearance rates is relatively less for stabilized formulations than for conventional liposomes [119, 120]. For neutral conventional liposomes, the window for optimal behavior is narrow, meaning that for effective application, liposomes should be small enough (preferably, <100 nm) but still maintain reasonable drug encapsulation efficiencies [83].

The EPR effect is a molecular weight-dependent phenomenon which does not exist in normal tissues [88]. Macromolecules larger than 40 kDa, which is the threshold of renal clearance, selectively leak out from tumoral vasculature and gradually accumulate in tumor tissues. Moreover, these accumulated macromolecules remained in tumor for a relatively long time.

Besides the size of liposomes, the clearance of liposomes is affected by their composition. Indeed, liposomal structure can significantly influence the stability and the drug release from liposomes [35]. The presence of cholesterol probably has one of the most important roles in the maintenance of membrane bilayer stability and long circulation times *in vivo*. Indeed, it was demonstrated that the presence of cholesterol and saturated phospholipids increased the rigidity of the lipidic bilayer and reduced the drug release. On the contrary, an excess of fluid lipid components gave to the liposomes the ability to easily break up and release the drug during circulation [35, 83]. An absence of cholesterol destabilized conventional liposomes by interactions with High Density Lipoprotein (HDL) [74] and

triggered the elimination of their components from the circulation. Pegaz *et al* evidenced an important difference of extravasation between Visudyne® and mTHPC loaded liposomes [121] in the model of the chick chorioallantoïc membrane. Visudyne® extravasates from CAM blood vessels very rapidly whereas mTHPC loaded in liposomes is confined into the vessels, at least during the first minute of observation. Those results indicated that the lipid composition of the liposomes governs their membrane fluidity and plays an important role in its behavior in the bloodstream. Indeed, mTHPC loaded liposomes composition is based on DPPC and DPPG whereas Visudyne® is based on more fluid lipids namely dimyristoylphosphatidylcholine and egg yolk phosphatidylglycerol (DMPC-EPG). This difference in composition explains the rapid extravasation of the Visudyne® compared to mTHPC loaded liposomes [122]. Thus, the lipid composition of the drug carrier, governing its membrane fluidity, has a very important incidence on the photosensitizer distribution in biological environment. The same principles were observed recently by Ytzhak *et al*, with hematoporphyrin embedded in liposomes with different lipid compositions, who found a correlation between the structure and unsaturation of lipids and the leakage of the plasmic membrane following photosensitization [123]. When liposomes were composed of a lipid mixture similar to that of natural plasmic membranes and photosensitization is being carried out under usual photodynamic therapy (PDT) conditions, photodamage to the lipids is not likely to cause enhanced permeability of ions through the membrane, which would have been a mechanism that leads to cell death.

The effect of liposome surface charge on liposome clearance kinetics is an increasingly misused predictive factor of circulation lifetimes [83]. Early studies have shown that the presence of negatively charged lipids in liposomes, including phosphatidic acid, phosphatidylserine and phosphatidylglycerol, results in a rapid uptake by the reticuloendothelial system [118, 124]. However, this relationship between the presence of charged lipids and circulation lifetimes is extremely complex and cannot be readily explained with simple models in which the presence of an anionic lipid necessitates increased clearance from the circulation [83]. Gabizon and Papahadjopoulos characterized the effect of surface charge on liposome clearance in mice using liposomes with different anionic phospholipids [125]. They noted that the inclusion of supplementary anionic lipids resulted in longer circulation lifetimes. Moreover, the vascular endothelial luminal surface is known to carry a negative charge which attracts positive charged basic proteins or cationic polymers in the proximity of vascular endothelial cells. This binding reaction leads to a reduced tumor drug accumulation by means of the EPR effect [126, 127].

2.3.2.2. Redistribution in Plasma

Liposomes present a short plasma half-life which is in a range of minutes [77]. Two different phenomena impair the circulation time of conventional liposomes. On the one hand, a lipid exchange between the liposomes and lipoproteins (especially High Density Lipoprotein, HDL) leads to an irreversible disintegration of the liposome with the subsequent release of the PS in the bloostream and its association with lipoproteins and others plasma proteins. On the other hand, conventional liposomes become easily opsonized by plasma proteins and taken up by cells of the mononuclear phagocyte system leading to a high liposomal concentration in organs and tissues with a rich mononuclear phagocyte system (liver, spleen, bone marrow) [75, 128]. As most tumor cells are reported to express an elevated number of LDL (Low Density Lipoproteins)-receptors due to their rapid

proliferation that increases the cholesterol demand for membrane synthesis [79], the final association with LDL might enhance the tumoral uptake of liposome-released photosensitizers by LDL-receptor-mediated endocytosis. In this context, the final lipoprotein association pattern of the PS, gradually released from disintegrating liposomes over a longer period of time, might vary signicantly from the pattern seen after a single bolus injection of the free PS [8, 75]. Indeed, different possibilities were evidenced dependently of physicochemical properties of the PS and plasma protein-binding behavior. As described above, free hydrophobic photosensitizers, associated with plasma proteins (LDL) to be transported in the circulation, can accumulate preferentially into both compartments via LDL-receptors-mediated endocytosis [129-131] or they can released from the PS-lipoprotein complex in the interstitial space and diffuse passively into tumor cells [132]. For hydrophilic photosensitizers, they can bind to albumin and HDL and be taken up by tumor cells via the nonspecific endocytosis pathway [133].

2.3.2.3. Reticuloendothelial System (RES)

The reticuloendothelial system (RES) can be described as a surveillance system for macromolecules. It is found in high quantity in organs like liver and spleen and can be a major obstacle to tumor delivery of macromolecular drugs. Many approaches based on surface modifications were explored to prevent phagocytic clearance by the RES. The most commonly used strategy is to conjugate PEG onto the surface of liposomes [88]. The PEGylation of liposome results in a hydrated barrier that provides good steric hindrance to the attachment of phagocytes. Moreover, PEGylation increases the circulation half-life of liposomes thus benefits EPR-based targeting of drugs to tumors [134]. These liposomes wearing modifications with glycolipids or PEGylated lipids are referred to as "sterically stabilized" liposomes. Alternatively, the term Stealth® liposomes has been proposed [75, 135].

2.3.2.4. Tumoral Microenvironment

2.3.2.4.1. Tumoral Lymphatic System

A lack of effective lymphatic drainage is usually evidenced in solid tumors [108, 113, 136-138]. In normal tissues, the lymphatic system has the ability to recognize macromolecules and lipid particles from the interstitial space. Impairment of the tumoral lymphatic system leads to a prolonged retention of liposomes in the tumor interstitial area [35]. Tumor interstitium is also characterized by a high interstitial pressure, leading to an outward convective interstitial fluid flow, as well as the absence of an anatomically well-defined functioning lymphatic network. Hence, the transport of an anticancer drug in the interstitium will be governed by the physiological and physicochemical properties of the interstitium and by the physicochemical properties of the molecule itself. In tumor tissues, not only there is an impaired lymphatic clearance but also the lymphatic system is the major route for metastasis of tumor cells in normal tissues [88, 104, 111].

2.3.2.4.2. Vascular Mediators

Different vascular factors are implicated in the EPR effect such as bradykinin, nitric oxide, VEGF, prostaglandins, collagenase, peroxynitrite, cytokines… [111]. A recent review

from Fang *et al* described with precision each of these factors [88] and their mode of action, particularly the process of extravasation of the macromolecules. EPR effect appears to be the result of multifactorial events *in vivo* which need to be deeply studied for the development of potential new strategies to modulate the EPR effect.

2.3.2.4.3. Heterogeneity of Tumors

Some parts of tumors, particularly the central area, do not exhibit the EPR effect and show less accumulation of macromolecules than other parts [88, 139]. Mice bearing tumors, which is the most frequently used experimental model, present necrotic and hypovascular areas proportionally extended to the size of the tumor. Consequently, the EPR effect, which is dependent on the neovascularization, does not present an homogenous pattern in the totality of the tumoral tissue.

2.3.2.4.4. Tumor Blood Vasculature

Contrary to normal tissues, solid tumors present a higher vascular density (hypervasculature) especially when tumors are small [88]. Tumor angiogenesis is described as one of the most important features that sustains rapid tumor growth via an angiogenesis stimulating factor: the Vascular Endothelial Growth Factor (VEGF) [140-142]. Indeed, fast-growing cancer cells demand the recruitment of new vessels or rerouting of existing vessels near the tumor mass to supply them with oxygen and nutrients [114]. The newly formed vessels have abnormal morphology characterized by defective endothelial cells with wide fenestrations of 100 to 600 nm [143], irregular vascular alignment, lack of a smooth muscle layer or innervation and a wide lumen [104, 109, 110, 136]. This disorganized morphology results from the imbalance of angiogenic regulators (VEGF) and matrix metalloproteinases [144]. Macromolecular transport pathways across tumor vessels occur via open gaps (interendothelial junctions and transendothelial channels), vesicular vacuolar organelles and fenestrations. Modifications of the blood flow behavior were also noted in the tumor vasculature. Indeed, blood flow was described as irregular and inconsistent in these vessels with frequent inversion of the flow direction [145]. These architectural and anatomical features of a tumor vascular system constitute the foundation of the EPR effect which leads to extravasation of macromolecular and lipid drugs. For the passively targeted liposomes, EPR is probably the only mechanism leading to selective tumor distribution [35].

2.4. Photoinduced Damage by Passive Targeting with Liposomes

2.4.1. Specific Models for Vasculature Visualization

Disruption of the neovasculature is known to play a crucial role in the eradication of tumors by PDT. Nevertheless, *in vitro* models can not give any information regarding vascular photothrombosis [146]. Vessel damage was first observed during the course of phototherapy in studies of Star *et al [147]*, Selman *et al [148]* and others [149]. The study of Star used a rat mammary tumor model grown in transparent sandwich chambers and allowed direct visualization of morphological changes in vessel physiology during and after PDT.

2.4.1.1. Window-Chamber Model (WCM)

It consist in a transparent window (1 cm in diameter) which is surgically placed into the dorsal skin of rodents (mice or rats) under general anesthesia. This allows direct visualization of the skin vasculature at high resolution under confocal microscopy [150-153]. As an example, De Visscher *et al* presented the window-chamber model installed in rats with mammary carcinoma transplanted in the subcutaneous tissue [154]. After intravenous injection, mTHPC fluorescence was detected with CCD camera in tumor tissue, vasculature and surrounding connective tissue. Just after injection, the vascular mTHPC fluorescence was high for Foscan® and Fospeg® but not for Foslip®. Each photosensitizer presented a different fluorescence intensity curve in time. Indeed, the maximum tumor fluorescence is reached at 48 hours for Foslip® and 24 hours for Foscan® and Fospeg®. Khurana *et al* used the same model but for a PDT treatment of AMD with Visudyne® in mice in order to demonstrate relationships between the PS quantification and light dose for a localized irradiation [150]. Even if this WCM model appears helpful, another model, offering a large variety of advantages, is actually in real development: the chick chorioallantoïc membrane model.

2.4.1.2. Chick Chorioallantoïc Membrane (CAM)

2.4.1.2.1. Description

The chick chorioallantoïc membrane assay is a commonly used method for studying angiogenic activities or drug delivery *in vivo* [155, 156]. It is a vascular membrane found in eggs of birds and reptiles. CAM corresponds to a transparent and highly vascularized membrane formed during the embryo development and results in a highly vascularized mesoderm composed of arteries, veins and an intricate capillary plexus [157]. First tumor transplantations to the CAM were described more than 100 years ago [158] but CAM tumor models remain sparse and poorly characterized as compared to murine models [158, 159]. The CAM assay is characterized by several major advantages such as the ease of access, the extensive vascularization and the relatively simple experimental approach and the natural immunodeficient environment of the developing embryo. This opens up the possibility to screen many samples in an inexpensive way [155]. Moreover, experiments in CAM do not need anesthesia contrary to the window-chamber model or standard rodents models. Until embryo development day 10, the chick embryo immune system remains incomplete with a lack of both B and T cell-mediated immune functions. Consequently, the young embryos are not fully immunocompetent and appear very appropriate to avoid the reject of xenografted cells or tissues.

The presence of T and B cells can be respectively first detected at EDD 11 and 12 [160]. After EDD 15, the B cell repertory begins to diversify and by EDD 18 chicken embryos become immunocompetent. It is essential to note that there are only 3 forms of immunoglobulins: IgM, IgG and IgA [161] in chicken compared to humans who possess 2 supplementary forms IgD and IgE.

Concerning the chick embryo culture, two approaches have been developed. An "*in ovo*" method where the embryos are left inside the eggshell during their development contrary to the "*ex ovo*" method where the embryos are cultivated in recipients simulating the eggshell (shell-less culture). The choice of the method depends of the age of the embryo and the nature of the intervention [156]. Administrations routes used with chick embryos are similar to those

used on humans: topical or intravenous (IV) [121, 122, 162] or intraperitoneal (IP) [163, 164]. Other routes as injection into the yolk sac [59] or in the amnion [165] can be used but without equivalence in humans.

2.4.1.2.2. Photodynamic Evaluation

The CAM model has been used to evaluate photosensitizers ability to induce vasculature damage. The photodynamic angioocclusion with Visudyne® was particularly well monitored by the team of Debefve *et al [166, 167]* in the model of the CAM. They detailed this process in real-time by using a CCD camera after an intravenous injection of the PS into the CAM. The effect of light activation was probed by the monitoring of an intravenous injection of a fluorescent dye (FITC-Dextran) 24 hours after PDT treatment. They observed a platelets accumulation at intravascular junctions within seconds after Visudyne® light activation and capillaries were closed 15 minutes later. An occlusion of the treated area was observed after 5 minutes with doses of Visudyne® and light similar to those used clinically. Other teams used the CAM model to test Visudyne® efficacy on vascular occlusion and demonstrated an improved therapy outcome [168, 169].

The team of Chin and colleagues worked with a topical application of Hypericin (HY) and Hypocrellin B (perylenequinones family) on human bladder cell line inoculated on the CAM [170]. Authors demonstrated a selective accumulation of both photosensitizers in the xenografted tumor and in the vasculature of the CAM together with very similar degrees of photoinduced damage to the CAM. More recently, the same team published a more detailed report on photoinduced cellular and vascular damage indicating that the tumor therapy based on targeting the vasculature of the tumor induced a higher relative regression percentage of treated tumor compared to cellular targeted PDT [98]. Saw *et al* published several articles on the transport of Hypericin formulations (NMP, N-methyl pyrrolidone 4.8 (High Dose group, HD) or 0.6 % (Low Dose group, LD) in CAM vasculature after topical administration [171-173]. The HD treated CAM demonstrated a vessel regression that was 2.37 times higher compared to LD treated CAM. One more time, the formulation considerably impacts on vasculature targeting PDT [172]. Hypericin–PDT effects showed to be HY and NMP concentrations-dependent and NMP appeared to be a promising solvent and penetration enhancer for HY-PDT [171]. Moreover, in the last study, NMP formulations produced significantly higher contrast for tumor tissues and permitted a more precise fluorescence diagnosis of human bladder cancer cells implanted in CAM [173].

Pegaz *et al* studied the photothrombic activity of mTHPC-loaded liposomal formulations in CAM [121]. They tested two different formulations: conventional liposomes (Foslip®) or the corresponding long circulating poly(ethylene glycol) (PEG)ylated liposomes (Fospeg®). The light dose necessary to induce the desired vascular damage with Foslip® was twice higher (100 J/cm²) than with Fospeg® (50 J/cm²) and offers PEG liposomes as a suitable delivery system for the treatment of choroidal neovascularization associated with AMD. Moreover, authors observed that mTHPC loaded in liposomes is confined into the vessels independently of the type of liposomal formulation. So, the PEG formulation did not significantly influence the extravasation of the PS.

Ismail *et al* examined the effect of PDT using topical methylene blue free and combined with liposomes as a PS for treating human ovarian malignant tumors cultivated on CAM [174]. Both formulations photoinduced a complete tumor remission from the surface of the CAM even

if the liposomal formulation induced higher fluorescence intensities in the tumor (accumulation in cell cytoplasm) and penetrated more deeply than the free PS. Free methylene blue had a vascular effect contrary to liposomal formulations which targeted the tumor with minimal effects on the surrounding and vascular tissues. Consequently, the authors proposed a combined treatment with free methylene blue as a debulking agent completed with another formulation (methylene blue-liposome) for the fine destruction of tumor remnants. Another comparative test of aqueous and liposomal formulations of Povidone-Iodine administrated onto the CAM revealed significantly less photoinduced angioirritative reactions of the CAM (coagulation, hemorrhages) with the liposomal formulation than with the aqueous formulation [175].

The large number of tumor models and PS loaded liposomes rend difficult the prediction of the PDT efficacy. Even if liposomal formulations were demonstrated to improve pharmacokinetics of PS, they not always improve their tumoral efficacy.

2.4.2. Liposomal Photosensitizers for PDT Efficacy

The majority of exogenous photosensitizers present a peak plasma concentration 5 minutes after intravenous administration followed by a fast exponentional decay in plasma drug level. The time period when the PS is confined into the tumoral vasculature (short DLI) provides a temporal therapeutic window for vascular targeting. PDT during this time period offers a great opportunity to potent vascular damage including blood cells, endothelial cells and vessel-supporting structures [9].

The primary pathway for the vascular effects of PDT most likely begins with initial damage to the vascular endothelial cells, leading to exposure of the vascular basement membrane and, thereby, to the creation of thrombogenic sites within the vessel lumen. This initiates a cascade of responses, including platelet aggregation, release of vasoactive molecules, leukocyte adhesion, and increases in vascular permeability and vessels constriction [129, 150, 176]. Based on the EPR effect, the therapeutic agents have also the capacity to accumulate in malignant tissues [107]. PSs used to passively target tumor cells should be macromolecules (above 40 kDa) and have a long circulation time. Indeed, it was demonstrated that tumor drug uptake is proportional to the drug circulation time [9]. After the extravasation of the PS into the interstitial space, the drug needs to be associated with tumor cell membranes or internalized into tumor cells to generate photocytotoxicity [9].

Different categories of liposomal photosensitizing agents are existing actually. Liposomal porphyrin, phtalocyanine, chlorin, bacteriochlorin and others “home-made” photosensitizers are described in the literature [35]. The passive photodynamic vascular-targeting approach is considered actually as the most successful PDT application and is used in current clinical context for the treatment of Age-Related Macular Degeneration (AMD) with Visudyne® (lipid formulation of benzoporphyrin derivative monoacid ring A (BPD-MA), Verteporfin). Visudyne® is the only photosensitizer which had received approval worldwide for AMD [177]. It corresponds to a unilamellar liposomal formulation improving its solubility [9]. The objective of the PDT treatment of AMD with Visudyne® is to shut down the abnormal choroidal neovasculature without damaging the normal retinal blood vessels. Richter *et al* compared liposomal formulation of BPD-MA to the free BPD-MA [8]. Authors demonstrated that both formulations had the same tumor and normal tissues distribution but the liposomal drug appeared to enter in the tissues more rapidly and to be cleared also more rapidly. However, liposomal BPD-MA presented a better efficacy in tumor damage after PDT

treatment with an intravenous injection and a DLI of 3 hours. The difference in PDT efficacy probably results from liposomal formulation allowing more BPD-MA to be associated with lipoproteins so that tumor cells can effectively take up the drug. Liposomal BPD-MA is also able to deliver BPD-MA to proliferating endothelial cells overexpressing LDL receptors [178]. Ishikawa *et al* [87] wanted to study the avoidance of reticuloendothelial system (RES)-trapping by using liposomal formulation of BPD-MA modified with polyethylene glycol (PEG-LipBPD-MA). The tumoral accumulation of BPD-MA in mice was significantly higher 3 hours after PEG-LipBPD-MA administration compared to non modified liposomal BPD-MA. On the contrary, tumor growth suppression was only observed following a PDT treatment with liposomal BPD-MA and not with PEG-LipBPD-MA. Thus, PEGylation enhances the passive targeting of liposomal BPD-MA in tumor but decreases the susceptibility of the drug in PDT. The explanation could be that liposomal carriers release very slowly BPD-MA at the tumor site. Furthermore, PEG in liposomes may protect direct interaction of liposomes with tumor cells whereas non-coated liposomes may contact more easily with tumor cells and deliver BPD-MA. PEG can also protect liposomes from interactions with macrophages in the interstitial space of tumors. In conclusion, although PEGylation of liposomes enhances its accumulation in tumor tissues, it does not always beneficial for therapy.

Actually, one of the most potent second generation photosensitizers is the meso-tetra(hydroxyphenyl)chlorin (mTHPC, Foscan®). It was approved for the palliative treatment of head and neck cancers. Generalized skin sensitization after systemic administration has been shown to be shorter as compared to porphyrin based photosensitizers [179]. Incorporation of mTHPC in lipid carriers has been proposed in order to improve pharmacokinetics, reduce systemic side effects as well as enhance specific drug delivery [180, 181]. Two formulations are actually used: conventional liposomes (Foslip®) or the corresponding long circulating poly(ethylene glycol) (PEG)ylated liposomes (Fospeg®). There are actually only few studies of the photodynamic treatment with Foslip® and/or Fospeg® realized *in vivo*. As an example, Buchholz *et al* compared the pharmacokinetics of Foscan® and Foslip® in a model of spontaneous feline squamous cell carcinoma in terms of tumor, skin and plasma pharmacokinetics [180]. They observed that the liposomal formulation of mTHPC had fluorescence intensities, fluorescence ratios (tumor /skin) and bioavailability in the tumor 2 to 4 times higher compared to free mTHPC. Moreover, maximal fluorescence intensity in the tumor was shown to occur 5.5 times earlier with liposomal mTHPC. Following this study, Buchholz and colleagues showed in the same model that this favorable pharmacokinetics of the liposomal drug resulted in a complete response rate of 100% [182]. The overall 1-year control rate was 75%. The tumor recurrence rate was 20% with a median time to recurrence of approximately 172 days. Once again, those results confirmed that lipidic formulations improve the pharmacokinetics properties of photosensitizers and consequently the PDT outcome. The same year, Svensson *et al* published a report which addressed Foslip® pharmacokinetics from 2 hours to 8 hours following intravenous administration in a murine model [183]. A rapid clearance from the plasma was noted and a noticeable average tumor/muscle ratio of 6.6 was evidenced. More recently, a Foslip®-PDT treatment of mice bearing subcutaneous tumors demonstrated 80% of tumor cures with a DLI of 6 hours which corresponded to an intratumoral vascular and parenchymal localization of the PS [181]. The highest tumor to muscle ratios were obtained at

6 hours and 15 hours after the intravenous injection of Foslip®. In the study of Westermann *et al*, the photodynamic treatment of human colon carcinoma xenografted in nude mice with Foscan® or Fospeg® 72 hours post-injection lead to the same potential to induce tumor regression although a longer blood circulating half-life and a better tumor to normal tissue ratio of mTHPC for the PEGylated formulation [184]. Another difference between Foscan® and Fospeg® was evidenced by fluorescence microscopy. Indeed, Fospeg® was localized near the tumor vessels whereas Foscan® more distributed inside the tumor tissue. Once again, this study confirms the possible improvement of pharmacokinetics of PS by their introduction in liposomes and PEGylation but the PDT outcome persists to be unpredictable in relation to the intratumoral distribution and dosimetry used during the treatment.

2.5. Impact of the Immune System

Pre-clinical research in various animal models has contributed immensely to our understanding of PDT-induced host immune responses as well as direct local cellular and vascular effects [185, 186]. Comparison of the antitumor effects of PDT in normal and immunodeficient mice revealed that despite comparable short-term outcomes complete antitumor responses were observed only in immunocompetent animals indicating that the engagement of the immune system is what makes PDT so effective [187, 188]. PDT has a significant effect on the immune system which can be either immunostimulatory or immunosuppressive [189, 190]. Most of the commonly used cancer therapies are immunosuppressive. Chemotherapy and ionizing radiations are delivered at important dose to destroy the tumor but are also known to be toxic for the bone marrow which produces all immune cells. Consequently, neutropaenia and other forms of myelosuppression are often the dose-limiting toxicity of these therapies [189]. Surgery can also have an immunosuppressive effect which triggers an important diminution of lymphocytes and natural killer cell function [191]. The ideal cancer therapy would destroy the tumor but also activate the immune system to recognize, track down and destroy any remaining tumor cells [189]. Different studies, both *in vitro* and *in vivo*, evaluated the relationship between the mode of tumor cell death and the efficiency of induction of the immune response. The majority of papers showed that cancer therapies which predominantly induced necrosis are actually better at activating the immune system than methods that predominantly induced apoptosis [192-194]. In fact, the necrosis corresponds to a liberation of cytosolic constituents into the extracellular space through the damaged plasma membrane which triggers a robust inflammatory response [189]. In the case of a tumor cell death by apoptosis, organelles are maintained isolated in apoptotic bodies which are phagocytosed by macrophages [195]. The acute inflammation induced by necrosis potentiates immunity by attracting host leukocytes into the tumor and increasing antigen presentation. PDT increases the immunogenicity of dead tumor cells by exposing or creating new antigens and by inducing heat-shock proteins (HSP) that increase the efficiency of antigen cross-presentation to form more effective tumor-specific cytotoxic T cells [189]. Particularly, the extracellular heat-shock protein 70 (HSP 70) was evidenced to be induced by PDT and released from necrotic tumor cells [187, 196, 197].

PDT can produce tumor cures and long-lasting tumor-specific immunity (memory) as has been shown by the rejection of tumors on re-challenge in certain mouse and rats models. One

of the first studies highlighting PDT-induced antitumor immunity in mice models was performed by Canti *et al* [198]. Authors worked with immunosuppressed and normal surviving mice that were previously treated with aluminium disulfonated phtalocyanines (AlS2Pc)-PDT. The re-challenge with MS2 fibrosarcoma tumor cells led to death of the immunosuppressed surviving mice while normal surviving mice tend to resist the re-challenge. The team of Korbelik *et al* is a particularly well known group for its work on the activation of the adaptative immunity necessary for a most effective tumor control [188, 199, 200]. Indeed, they demonstrated that an adoptive transfer of bone marrow cells [188] or lymphocytes [199] from immunocompetent to immunodeficient animals allowed a complete restoration of the curative antitumor effects of PDT. It was also shown that PDT has the ability to instigate antitumor T-cell specific immunity which also leads to generation of immune memory cells that are recoverable from distant sites [186]. Those experiments illustrated perfectly the importance of the immune system for the success of the photodynamic treatment.

Photodynamic therapy was presented here as many studies described it: a treatment which has the ability to induce irreversible damages to pathological tissues, via vascular shutdown and induction of cell death pathways, specifically described for different types of cancers. Another facet of the PDT treatment is its potential to improve the regeneration of tissues. Immune system always plays a very important role and we are going to present the interest of the PDT to promote the wound healing process, particularly in the skin.

3. Photoinduced Tissue Regeneration by PDT

3.1. Wound Healing Definition and Description

Wound healing is a highly orchestrated process which requires interactions between soluble mediators, extracellular matrix components, resident and hematopoetic cells, as keratinocytes, fibroblasts, endothelial cells, nerve cells and infiltrating leukocyte subtypes as neutrophils, macrophages, mast cells and lymphocytes. Each of these components interferes differentially in the classically defined three phases of the wound healing that overlap in time and space: inflammation, tissue formation and tissue remodeling [201, 202].

Phase 1: Inflammation. Inflammation occurs immediately after tissue damage. Components of the coagulation cascade, inflammatory pathways and immune system are enrolled to prevent ongoing blood and fluid losses and to remove dead and devitalized tissues to prevent infection. Haemostasis is achieved progessively by the initial formation of a platelet plug followed by a fibrin matrix which will be employed as a scaffold for infiltrating cells [203]. Activation of the complement, degranulation of platelets and products coming from bacterial degradaton permit to recuit neutrophils. Two to three days later, monocytes emigrate from blood into the wound and differenciate into macrophages [203].

Phase 2: Tissue formation. This step occurs 2-10 days after injury and is characterized by cellular proliferation and migration of different cell types [203]. In tissue formation, epithelialization and newly formed granulation tissue, consisting of endothelial cells,

macrophages and fibroblasts, begin to cover and fill the wound area to restore tissue integrity [204]. Fibrin, fibronectin, vitronectin and tenascin are components of the provisional extracellular wound matrix which facilitates cell adhesion, migration and proliferation [205]. At the onset of Phase 2, keratinocytes migrate over the injured dermis (re-epithelialization) and new blood vessels are formed by angiogenesis process. The association of neovessels, fibroblasts and macrophages replace the fibrin matrix with granulation tissue. The latter can be used as a substrate for the migration of keratinocytes which will proliferate and mature to restore the barrier function of the epithelium at later stages of the repair process. Once the denuded wound surface has been covered by a monolayer of keratinocytes, epidermal migration ceases and a new stratified epidermis with underlying basal lamina is re-established from the margins of the wound inward [205].

Phase 3: Tissue remodeling. This phase starts 2-3 weeks after injury and can lasts one year or more. Most of the processes engaged precedently cease and different cell types as endothelial cells, macrophages and myofibroblasts die by apoptosis or exit from the wound. Only a mass composed by few cells, collagen fibers and extracellular matrix proteins persist at the previous wound site [203]. During this last phase, a balance is reached between synthesis of new components of scar matrix and their degradation by proteases. The mechanisms determining granulation tissue regression and its transformation into scar tissue is not well understood. Even if typical features of these events are evidenced (regression of vascular structures, maturation of fibroblasts into myofibroblasts, resolution of the inflammatory response…), the balance between the stimulation of anti-inflammatory mediators and the downregulation of proinflammatory factors has not been clearly identified [205].

3.2. Factors Involved in Wound Healing

The major steps constituting wound repair remain actually poorly characterized at the molecular level. Nevertheless, important roles of some cells and factors have been highlighted.

3.2.1. Cell Lineages

Polymorphonuclear leukocytes (PMN). The early inflammatory phase or repair is characterized by local activation of the innate immune system, resulting in an early influx of polymorphonuclear leukocytes (neutrophils, PMN) followed by invasion of blood monocytes which differenciate into tissue macrophages [205]. Immediately after injury, extravasated blood constituents form a hemostatic plug. Platelets and PMN, entrapped and aggregated in the blood clot, release a wide variety of factors which are responsible of the amplification of the aggregation response, a coagulation cascade and can act as chemoattractants for cells involved in the inflammatory phase [206]. Few hours post-injury, neutrophils transmigrate across the endothelial cell wall of blood capillaries previously activated by proinflammatory cytokines IL-1β, tumor necrosis factor (TNF-α) and interferon (IFN-γ). These factors lead to the expression of different adhesion molecules: P- and E-selectin, ICAM-1 and -2, essential for leukocyte adhesion and diapedesis. Different studies demonstrated, in mice deficient for P and/or E selectins or ICAM-1, a dramatic delay in wound closure in relation with a decrease of

macrophages infiltration [207, 208]. Indeed, adhesins interact with integrins present at the cell surface of neutrophils (CD11a/CD18, CD11b/CD18, CD11c/CD18, CD11d/CD18) [209]. Chemokines and their receptors play a crucial role for neutrophil recruitment during wound repair [201]. Engelhardt *et al* particulary evidenced IL-8, the growth-related oncogene α and the monocyte chemoattractant protein-1 (MCP-1). Moreover, bacterial products in the wound area can accelerate the neutrophil locomotion. Finally, recruited neutrophils realize the debridement of devitalized tissue and phagocytosis of infectious agents by releasing highly active antimicrobial substances (reactive oxygen species, cationic peptides, eicosanoids) and proteases (elastase, proteinase-3, cathepsin G…) [204, 205].

Monocytes/Macrophages. Beside their functions as antigen presenting cells and phagocytes, macrophages play a central role in wound repair due to their capacity to synthesize inflammatory cytokines and growth factors, such as Transforming Growth Factors α-β (TGF α-β), basic Fibroblast Growth Factor (bFGF), Platelet Derived Growth Factor (PDGF) and Vascular Endothelial Growth Factor (VEGF) [205, 210]. Monocytes emigrate from blood into the wound by following gradients of chemoattractic factors like growth factors, proinflammatory cytokines, chemokines macrophage inflammatory proteins (MIP-1α, MCP-1, RANTES) [211, 212]. After a skin injury, the neutrophil infiltration halts after few days and neutrophils are phagocytosed by macrophages recruited from the blood 2 days after injury.

Mast cells. Mast cells are members of leukocytes cells represented in most tissues. They are able to synthesize an important quantity of proinflammatory mediators and cytokines which promote inflammation and vascular changes [204, 205, 213, 214] Contradiction results on the role of mast cells have been obtained up to now [213-215]. Indeed, some teams reported an absence or a poor impact of mast cell-deficiency on skin repair, particularly on re-epithelialization, collagen synthesis and angiogenesis [216, 217] On the contrary, a more recent study evidenced a significant impact of mast cell-deficiency on vascular permeability and PMN influx [218]. Noli *et al* reported the potential detrimental effect of an excessive and uncontrolled mediator release (hyperdegranulation). The repair response appears to be highly dependent of the model system used to clarify the role of mast cells. Particularly, mutant mice present abnormalities which might influence the wound healing process and genetic aspects need to be identify.

T cells. T cells are another leukocyte subset observed in human skin wounds during the phase of tissue remodeling when wound closure is completed and local infections overcome. Lymphocyte chemotaxis is highly dependent on chemokines, in particular interferon-γ-inducible protein-10 (IP-10) and the monokine induced by interferon -γ (MIG), both provided by macrophages. T cells can also influence the healing response by direct cell-cell interactions with resident (keratinocytes, fibroblasts) and non resident (platelets, macrophages) cells at the wound site [204, 205].

3.2.2. Growth Factors

Some different factors interfere in the wound repair process, particularly during the second phase of tissue formation [203, 211]. Indeed, the re-epithelialization phase needs the

intervention of the hepatocyte growth factor (HGF) by linking with the receptor tyrosine kinase MET. *Chmielowiec et al* demonstrated that cells which were deficient in the protein MET can not contribute to the formation of a neo-epidermis [219]. Other growth factors like members of the FGF (fibroblast growth factor) or EGF (endothelial growth factor) family are involved in re-epithelialization but have not the capacity to compensate a lack of HGF mediated signaling. On the other side, certain growth factors can negatively regulate wound re-epithelialization. For example, it was shown that mice expressing a gene encoding a dominant negative TGF-β receptor in the epidermis presented a strong acceleration of the re-epithelialization [220]. Concerning angiogenesis, the most important promoting factors are vascular endothelial growth factor A (VEGF-A) and fibroblast growth factor 2 (FGF-2 or bFGF). For example, the administration of VEGF-A on wounds observed in a diabetic animal model permitted to restore a normalized healing [221, 222].

3.2.3. Extracellular Matrix Components

Re-epithelialization process needs the intervention of an important quantity of proteins among which various extracellular-matrix proteins and their receptors, proteases (matrix metalloproteinases), cytoskeletal proteins and enzymes which regulate the cellular redox balance [223]. Numerous studies have demonstrated that the composition of the underlying extracellular matrix can modify the rate of keratinocyte migration [224, 225]. The most important constituent of the extracellular matrix is the collagen. During the initial phase of wound healing, type III collagen and fibronectin are deposited and later type III collagen will be replaced by type I collagen which promotes keratinocytes migration. It is likely that type IV collagen contributes to this effect but the action mechanism is not well known [226]. Fibronectin, laminin and vitronectin are others components identified as factors for promoting keratinocytes migration [226-229]. Matrix metalloproteinases are essential for the re-epithelialization because they degrade extracellular matrix thus removing both damaged tissue and provisional matrices and allowing cell migration [226].

In conclusion, understanding the network of wound healing involves a profound analysis of all soluble mediators and adhesion factors essential in the recruitment and trafficking of leukocytes during the inflammatory reaction. Cellular responses to injury involve direct cell-cell and cell-matrix interactions as well as the indirect crosstalk between different cell populations by soluble mediators. Cutaneous wound healing implicates complex and essential interactions between the epidermal and dermal compartments. An important quantity of factors have been identified by their action between epidermal and dermal cells to facilitate wound repair [211]. There is a crucial and sensitive balance between stimulating and inhibiting mediators during wound healing process in order to achieve tissue homeostasis following injury [211].

3.3. Clinical Context

In humans, wound healing problems can be translated by two opposite observations: either a delayed wound healing, which occurs with diabetes or radiation exposure, or an excessive wound healing observed with hypertrophic and keloïd scars [230]. Excessive healing is constated by a large deposition of extracellular matrix and alterations in local vascularization and cell proliferation [203]. Intralesional steroids are the most effective and widely used

treatment for keloïds. Surgical excision of keloïds generally results in recurrence rate of keloïds related to a stimulation of an additional collagen synthesis resulting in rapid regrowth and often a larger keloïd [230]. Administration of single agents therapies like growth factors [231, 232] was tested but not led to substantial advances in patient care probably related to the rapid degradation of this components at the wound site. Radiation therapy has been shown to effectively reduce the recurrence rate of keloïds by directly damaging fibroblasts altering collagen structure and organization [230]. The more recent proposition to both overhealing and underhealing is the administration of cells with the ability to produce all components necessary to regulate the microenvironment of the wound area [203].

Other parameters need to be taken in account for the study of wound healing process. Indeed, the age of the subject, the nutrition [233, 234], the immune state, the local vascularization and hypoxia of the wound area [223], the stress [235] are parameters which can influence dramatically wound healing. For example, anoxia is a contributor to non healing wounds and increasing delivery of oxygen to tissue is one clinical approach to enhancing wound repair. The changes in oxygen tension could initiate, as yet unclear signaling cascades that culminate in proliferation and increased collagen production [236]. Over the past decade, it has become clear that stress can significantly slow down wound healing. It can have different origins as glucocorticoïds or proinflammatory cytokines and it can induce substantial tissue hypoxia responsible of a lack of wound healing.

3.4. PDT and Wound Healing: the Pros and Cons

Whereas PDT is mainly applied in oncologic settings, it has also widely been used to target vasculature in age related macular degeneration, and is now under investigation for its microbial activity, especially in wounds or peridontitis [237]. In the following paragraphs, PDT effects on wound healing are presented by their opposite actions: inhibition versus promotion of healing response.

3.4.1. Nefast Effects on Wound Repair

Only few studies exposing negative effects of PDT on wound healing have been published. Different studies in animal models have demonstrated that PDT has significant impact on wound healing, including delay of early granulation tissue formation, delay in re-epithelialization, necrosis of muscle and mucosal tissues [238]. One laboratory had particularly insisted on those negative aspects [239-241]. They used PDT as an adjuvant intraoperative treatment to improve locoregional control specifically on revascularization of a rat fasciocutaneous flap. Indeed, initial free vascular flaps are dependent on their vascular pedicle and long-term survival is facilitated by revascularization of the surrounding tissue bed. By using 5 mg/kg of Photofrin® and fluences at 25, 50 and 75 J/cm², Kubler *et al* showed delayed wound healing, causing effusions, discoloration of the flaps, necrosis, scab formation, lower tensile strength and a prolonged and more widespread inflammatory reaction in pedicled flaps when the operative bed was treated by PDT [240, 241]. Authors insisted on the fact that their rat model is extremely sensitive, both systemically and locally, to the effects of PDT. That's why it appears essentially to choose with precautions the animal model and compare different models between them before to experiment. In their last study, authors

realized a fasciocutaneous flap on rats and treated them by Photofrin®-PDT with two different primary ischemic times: 2 or 4 hours. With 2 hours of ischemia, a decrease of the revascularization was observed in the PDT treated group on postoperative days (POD) 6 and 7. With 4 hours of ischemia, the decrease appeared already at POD 5, 6 and 7 only in the PDT treated group. PDT has been shown both to delay wound healing and to have a deleterious effect on flap survival after a primary ischemic insult. This delay may make the flap dependent on its pedicled blood supply for a prolonged period [239]. Another article, published later, sought to evaluate Photochlor (HPPH) photodynamic therapy (HPPH-PDT) as an adjuvant therapy to prevent recurrence of tumor after surgical removal in a model of canine hemangiopericytomas. Photochlor was injected intravenously at a dose of 0.3 mg/kg. Forty-eight hours later the treatment consisted of surgical removal of the tumor followed by HPPH-PDT. Photochlor photodynamic therapy applied after surgery appears to have no advantage over other forms of therapy in regards to preventing recurrence. Delayed wound healing and infections are problematic and make HPPH-PDT an undesirable addition to surgery for the treatment of this tumor type [242].

Some teams revealed also an absence of PDT effect on wound healing [243-245]. As an example, Parekh *et al* observed that PDT after systemic administration of either benzoporphyrin derivative (BPD-MA) or chloraluminium sulphophtalocyanin (CASP) had no influence on rate or final appearance of wound healing as observed after pathological analysis two weeks later [244]. They also precise that even if the rat offers a good model for detecting inhibition of wound healing, any acceleration or improvement in wound healing would be difficult to detect. So, the absence of effect could be an undetected effect. Once again, the type of animal model seems critical. Furthermore, previous studies have suggested that laser light alone benefits wound healing at low powers [246-248] and delays the healing process at high powers [249]. The use of low-level light therapy (LLLT) has been advocated, however it is highly contradictory in humans as well as in rodent models. *In vitro* and *in vivo* studies as well as human clinical trials show antagonistic results [250].

3.4.2. Improvements of Wound Repair

Majority of papers concerning PDT and wound healing let appeared a promoting effect of the treatment during various phases of the healing process.

3.4.2.1. Re-epithelialization and Re-endothelialization

Different studies have suggested a promotive influence of PDT on wound healing in rats in terms of reepithelialization and remodeling [251-255]. Skin illumination to perform PDT after systemic administration of a photosensitizer is known to induce skin necrosis, however low doses light appeared to fasten the process of wound healing [253]. Jayasree *et al* administered aminolevulinic acid (ALA) intraperitoneally or hematoporphyrin derivative orally in rats, followed by HeNe and or NdYAG illumination [253]. They noted a clearly improved macros and microscopic healing after ALA based PDT, without however any effect on tensile strength at 3 weeks post injury. Particularly, they constated a re-epithelialization by day 15 in treated groups versus not in controls, mature collagen and reduced wound width at day 21 post-PDT versus immature collagen in controls. Other recent studies also reported that topical application of a photosensitizer and repeated illumination improved wound healing [252, 255]. Indeed, rat punch biopsy wounds treated with local administration of phtalocyanines followed by daily

illumination at low light dose for 1 week presented a faster healing: an enhanced collagen content and matrix remodelling and greater epithelial regeneration [255]. Topical application of toluidine blue after punch biopsy in rats third-degree burns, followed by light only or PDT also improved healing as compared to controls. An increased collagen and intense angiogenesis were observed on day 3 conducting to an intense and parallel and organized collagen and epithelialization on day 7. The difference observed at day 14 between the 2 groups (PDT versus control) mainly consisted in a 100 % epidermal regeneration for PDT as opposed to 50 % in the control group [252]. The beneficial effects of PDT were attributed to both the low level illumination, and the bactericidal effect of PDT. For Adili *et al*, rat carotid injuries treated by Verteporfin®-PDT presented an increased endothelial cell lining 5 and 14 days after PDT, a higher proliferation on PDT-matrix and an increased bFGF-mRNA quantified by PCR [251]. More recently, Reddy *et al* [254] used methyl aminolevulinic acid photodynamic therapy (MAL-PDT) on patients with superficial and nodular basal cell carcinomas (BCCs). Two separated treatments at 7 days interval were administered as adjunctive treatment for Mohs micrographic surgery. A more rapid re-epithelialization of wounds and decreased scarring response was observed after PDT. In the majority of the papers, re-epithelialization is the first process which appeared to be promoted by PDT without however clear explanations about molecular processes engaged in the reaction.

3.4.2.2. Fibroblasts and Myofibroblasts

Even if the re-epithelialization is the principal studied aspect in wound healing, the migration of fibroblasts and myofibroblasts during the remodelling phase can be modulated by PDT treatment. Stasi *et al* presented a model of a rabbit glaucoma treated with sclerectomy and Verteporfin®-PDT [256]. The treatment triggered a decrease of the fibroproliferative response compare to control group, confirmed by histology. An increase of granulation tissue with abundant endothelial channels that allowed aqueous humour filtering to the bleb was also noted. This result indicated that the aggressive wound healing in this rabbit model of glaucoma surgery, chosen specifically for this particularity, can be significantly delayed by the use of PDT with intravenous photosensitizer administered postoperatively. The team of Adili *et al*, already cited in the previous paragraph, promoted a decreased myofibroblast migration and consequently a favourable healing with a model of rat carotid injuries treated by methylene blue-PDT [257]. The potential of PDT to limit (myo)fibroblasts migration during the wound healing process avoid the formation of fibrosis. Mechanisms of interaction between PDT and fibroblasts are not yet identified.

3.4.2.3. Antimicrobial Action

Infections are widely known to perturb the wound healing process by delaying the re-epithelialization due to bacterial proliferation. At the beginning, PDT of wound infections has not been explored probably due to inefficient photosensitizer, a lack of selectivity for prokaryotic cells and the difficulties inherent in monitoring the response of localized infections in small rodents [258]. In recent years, antimicrobial effects of PDT recovered interest and was proposed as a therapy for a large variety of localized infections. PDT has been successfully used to kill pathogens and even to save life in several animal models of localized infections such as surface wounds, burns, oral sites, abscesses and the middle ear [237]. PDT presents several advantages compared to other standard therapies. Indeed, no

pathogens resistance was demonstrated after PDT, the treatment can be used for non-perfused tissue (burns) and is less toxic compared to others [259].

Hamblin *et al* were the first team to report the use of mouse wound infection models to investigate the effect of PDT on treating excisional wounds infected with *Escherichia coli* and *Pseudomonas aeruginosa [258, 260]*. The experiments consisted in single wounds (100 mm^2) realized on the backs of healthy mice and infected with a suspension of bioluminescent bacteria transduced with a plasmid containing a bacterial lux gene operon which permitted to monitor the infection in real time by a sensitive charge-coupled camera. Wounds were treated one hour later by a topical polycationic photosensitizer conjugate at 160 J/cm^2 in four 40 J/cm^2 aliquots with imaging taking place after each aliquot [258]. The PDT treated group led to a 99% reduction in luminescence. Moreover, authors observed that PDT of infected wounds did not lead to any inhibition of wound healing which can be explained by the combination of the topical delivery method together with the large conjugate size and the relatively short incubation time [258]. In their following study, authors used the same model and observed that with a *P.aeruginosa* infection all non-treated mice died within 5 days contrary to PDT-treated group where 90% of mice survived [260]. PDT treated wounds healed significantly faster compared to other groups and authors believe that the explanation lies in the ability of topical PDT to inactivate extracellular virulence factors (proteases, lipases, toxins and siderophores) which are abundantly expressed by *P. aeruginosa* and have been proposed to aid in bacterial invasion and tissue damage [260, 261]. Using similar mouse models, Wong *et al* and Zolfaghari *et al* studied the effect of methylene blue and toluidine blue O mediated PDT on *Vibrio vulnificus* and methicillin-resistant *S. aureus* wound infections. A significant reduction of bacterial numbers was observed in both studies and PDT can cure mice with otherwise fatal *V. vulnificus* wound infections [262, 263].

3.4.2.4. Immunomodulation

The role of PDT in the stimulation of the host immune system is an important topic which has not so far been much investigated for infectious disease [237]. It was already demonstrated that PDT has the capacity to increase the host immune response against the cancer [187, 189, 264]. Indeed, the photo-induced direct damage of neoplastic cells, creating and releasing a mixture of tumor antigens and cellular danger signals, triggers an acute inflammatory response which activates and matures dendritic cells and other cellular components of both the innate and adaptive immune systems [189].

In principle, we could apply the same process for the improvement of wound healing but actually, according to our knowledge, the relation with wound healing was not described. The impact of PDT on immune response in wounds infections stays an undiscovered field of research for the future and dramatically needs deeper investigations.

Conclusion

As described in this review, the Photodynamic Therapy efficacy is based on the combined action of three essential parameters: the photosensitizer, the light and oxygen. On the first hand, it is possible to exploit the PDT ability to inhibit proliferative evolution of pathological cells, particularly in the treatment of cancerous diseases. On the other hand, PDT can also have an opposite effect, promoting the tissue repair by the stimulation of growth

factors synthesis. Even if these two opposite aspects of PDT implicate the same parameters, underlined mechanisms are completely different. The predominance of each effect (photoinduced damaging or repair) will depend on the complex relationship between different parameters (fluence, fluence rate, drug delivery…) implicated in phototherapy and as such their comprehension is essential.

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Chapter 2

Nanostructured Third Generation Photosensitizers for Anticancer Photodynamic Therapy

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Abstract

Photodynamic therapy (PDT) is an approved cancer treatment in the USA, Brazil, China, and several countries in the European Union. It has been successfully used as an alternative treatment for superficial malignant tumors, such as skin, esophagus, and oral cancer. The basis of this therapy is a photochemical reaction promoted by photosensitizers (PS) when irradiated with light at specific wavelengths. This photoactivation, in presence of oxygen, induce the production of a series of reactive oxygen species (ROS), which are the cytotoxic agents involved in the elimination of cancer cells. In despite of the good clinical outcomes presented by the existing PS, some important drawbacks still remain to be solved. The 1st generation porphyrins show prolonged skin phototoxicity, extended retention in the host organism, low extinction coefficient and absorption peak at short wavelengths. The 2nd generation porphyrins present improved characteristics in comparison to the 1st generation PS, but still show inconvenient pharmacokinetics properties and loss of photodynamic activity in aqueous media. In order to overcome some of these drawbacks, the association of these PS molecules to specific carriers has been suggested by some researchers. This strategy resulted in the 3rd generation porphyrins, which show improved activity against malignant tumors. A brief review of the literature shows that nanostructures are increasingly being used as carriers for the development of 3rd generation PS. In this chapter we will present a review of the most important nanostructured drug delivery systems used as carriers for PS in the field of anticancer PDT.

* All the authors contributed equally to the writing and the critical analysis of this chapter.

1. Introduction

Photodynamic therapy (PDT) is approved for cancer treatment in USA, Brazil, China and several countries in the European Union. It has been successfully used as an alternative treatment for superficial malignant tumors [1]. PDT is based on the products of photoreactions triggered by light excitation of photosensitizers [2]. The photoreactions involved in PDT are represented by the Jablonski diagram (Figure 1). When irradiated with light at a specific wavelength, the photosensitizer (PS) is excited from its singlet ground (S_0) state to its first excited singlet (S_1) state.

The PS at its S_1 state can emit a photon by the phenomenon of fluorescence, react with surrounding molecules, or reach its first excited triplet (T_1) state through intersystem crossing (ISC). The PS at its T_1 state can emit a photon by phosphorescence, react with surrounding molecules or specifically interact with triplet oxygen exciting it to its first excited singlet state. In oxygenated media the T_1 state PS readily interacts with triplet oxygen producing singlet oxygen [2].

The photoreactions involved in PDT are classified as Type I and Type II, accordingly to their photophysical and photochemical mechanisms. In Type I photoreactions, several molecules can be directly oxidized by the excited PS [2]. The reduced PS may then react with molecular oxygen, generating superoxide anion and its derivative series of reactive oxygen species [2-4]. In Type II photoreactions, the PS works as a photocatalyzer for the production of singlet oxygen, instead of being a reactant.

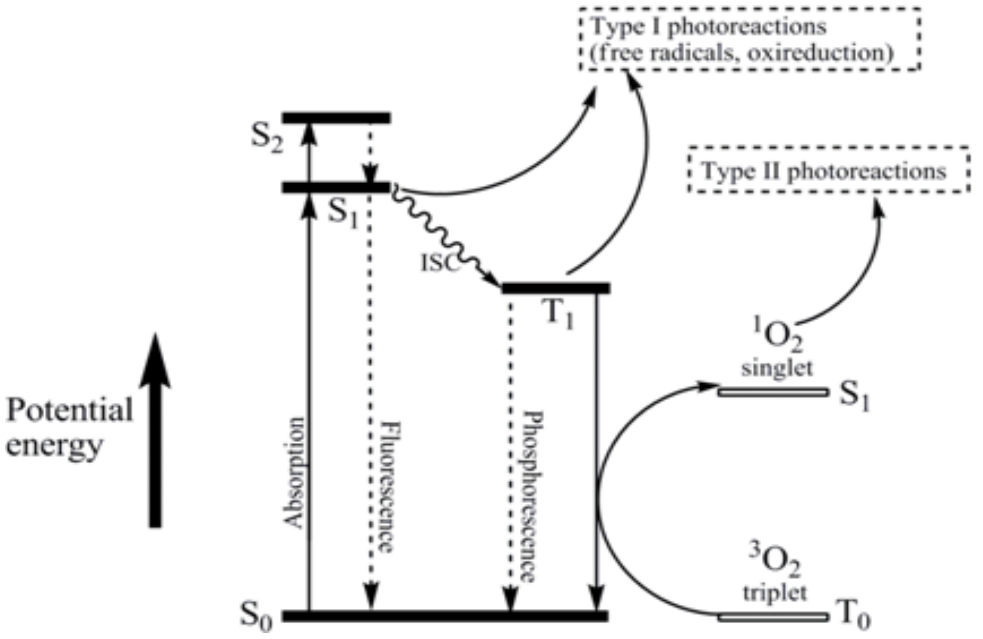

Figure 1. Jablonski diagram showing the photophysical events involved in Type I and Type II photoreactions. Full horizontal black lines represent the energy levels of a photosensitizer molecule; empty horizontal lines represent the energy levels of molecular oxygen. S_0 = ground singlet state; S_1 = first excited singlet state; S_2 = second excited singlet state; T_0 = ground triplet state; T_1 = first excited triplet state; ISC = intersystem crossing. Absorption and fluorescence/phosphorescence refer to absorption or emission of photons, respectively.

Although both Type I and Type II photoreactions account to the final effect of the PDT, the second one is often predominant in aerobic media [2]. The basic components necessary for Type II photoreactions to occur are the triplet oxygen, light of a specific wavelength and a PS [4]. The production of singlet oxygen through the photoreaction catalyzed by a PS can be diagramed as presented in Figure 2. It is worth noting that, as singlet oxygen diffuse only through few nanometers in biological media [5], the main effects of PDT are restricted to the site where both the light and the PS are concentrated during the application of this therapeutical protocol.

Since singlet oxygen is a highly oxidant oxygen species, it reacts with most of the organic biological molecules and is therefore a strong cytotoxic agent [6]. However, in anticancer PDT, the direct oxidative damage promoted in cancer cells is not the only responsible for their elimination. The anticancer activity of PDT depends on three biological mechanisms (Figure 3) following photoreactions [7]: 1) cancer cell death following oxidative stress; 2) tumor vasculature occlusion; 3) activation or boosting of anticancer immunity.

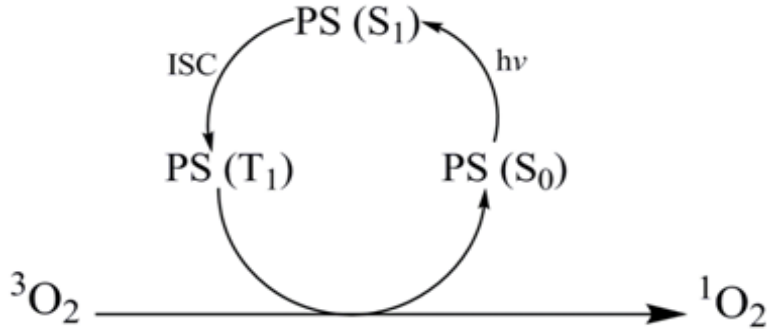

Figure 2. Diagram showing the photosensitizer as a photocatalyzer of the conversion of triplet oxygen ($^3O_2$) to singlet oxygen ($^1O_2$). PS = photosensitizer; S_0 = ground singlet state; S_1 = first excited singlet state; T_1 = first excited triplet state; ISC = intersystem crossing.

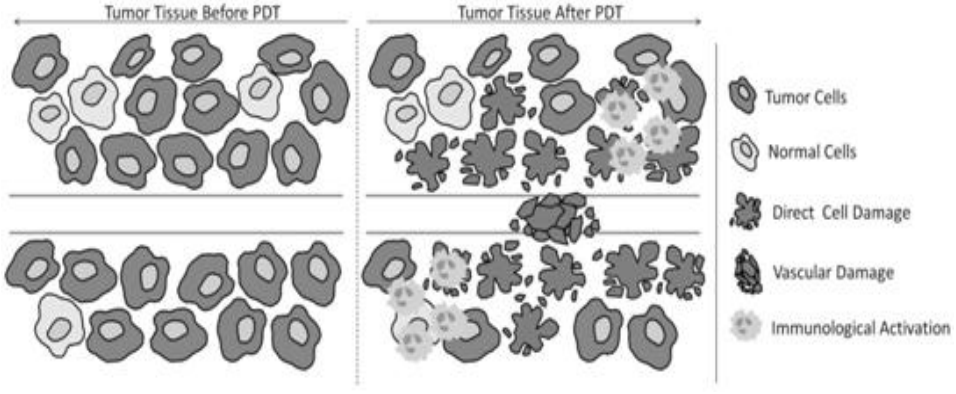

Figure 3. Schematic representation of the general effects of photodynamic therapy on malignant tumor tissue. After the application of photodynamic therapy, the cancer cells can be eliminated through three different processes: 1) direct oxidative damage in cancer cells; 2) obstruction of blood flow following vascular damage; 3) activation or boosting of an immune response against cancer cells.

2. Photosensitizers for Anticancer Photodynamic Therapy

As exposed in the precedent session, the three pillars of PDT are the photosensitizer, the molecular oxygen and light at a certain wavelength. Therefore, these three factors crucially affect the outcome of anticancer PDT. In the field of medicinal chemistry and pharmaceutics for PDT, however, the interest lies on the development and improvement of PS molecules and PS carriers.

Generically, PS are compounds that promote photoreactions under light excitation. An ideal anticancer PS should present the following characteristics [4, 8, 9]: 1) be chemically pure; 2) be minimally toxic in the absence of light; 3) be preferentially retained by the target tissue; 4) present a short half-life in the body to prevent side-effects related to extended retention in non-target tissues; 5) be active in aqueous media; 6) strongly absorb light at wavelengths between 600 and 800 nm; 7) present a high quantum yield for the generation of singlet oxygen; and 8) be photostable. These characteristics are mainly related to or limited by: 1) efficient photogeneration of singlet oxygen; 2) optical properties of biological tissues; 3) interaction with biological systems, and 4) tumor selectivity. Since the first studies on anticancer PDT, more than a century ago, several PS were described in the literature. These PS are generically classified as porphyrinoids and nonporphyrinoids, accordingly to their chemical structure [1].

The nonporhyrinoid PS are diverse in relation to their chemical structure, sharing only the presence of multiple conjugated π bounds. This class of PS comprises several kinds of molecules, including some dyes, such as cyanines and phenothiazinium dyes, and naturally occurring compounds, such as polycyclic quinones [10]. Nonporphyrinoid PS were found to present interesting properties for anticancer PDT, as reviewed elsewhere [10]. However, the development of this class for its application in oncology is considerably delayed in comparison to the porphyrinoid one.

Porphyrinoid PS have in common a carbon macrocycle comprising 18 to 22 electrons in conjugated π bounds [1]. This class comprises the most important PS molecules used in PDT nowadays. Since the seminal studies on hematoporphyrin – the first porphyrinoid PS described in the literature [10] – researchers have found several porphyrinoid PS useful for PDT. Therefore, according to their characteristics and chronological aspects, porphyrinoid PS are further classified as first, second and third generation.

The first generation of porphyrinoid PS comprises hematoporphyrin, hematoporphyrin derivative and Photofrin® (also known as pofirmer sodium). All of them are mixtures of porphyrins obtained from blood after chemical modification and purification steps. This class is important not only for historical reasons, but also because Photofrin® is considered as the gold-standard PS for PDT [10]. Photofrin® is approved for the treatment of several kinds of cancer [5].

Despite good therapeutical performance and clinical approvals, the first generation PS present important drawbacks, mainly [8, 10-12]: 1) lack of chemical purity, since they actually are a variable mixture of different compounds; 2) long half-life times and intense dermal retention, responsible for a prolonged dermal photosensitization (2 to 3 months); 3) low molar extinction coefficients, and 4) peak of absorption of light at short wavelengths (near 600 nm). These drawbacks induced a search for new PS molecules. This investigation

led to the discover of several new PS molecules, which present better characteristics for PDT in comparison to the first generation PS [10]. These molecules are classified as second generation PS.

However, the second generation PS are still very hydrophobic and poorly selective for tumors. Particularly, the problem of low selectivity of the available PS led scientists to focus on the development of PS carriers for tumor-targeting. The main targeting strategy employed has been the conjugation of PS molecules to tumor-specific carriers, such as certain antibodies and nano-sized materials [1, 13]. These PS compositions with improved tumor selectivity are classified as third generation PS and are a topic of intense ongoing research. The following sessions focus on the main aspects of nanostructured drug delivery systems and on the nanostructured third generation PS for anticancer PDT.

3. General Aspects of Nanostructured Drug Delivery Systems for Anticancer Therapy

The term "nano-" – derived from the Greek word for dwarf – is a prefix meaning one-billionth ($1/10^9$). Nanoscience and nanotechnology refer to science and technology that study and manipulate the matter at the nano-scale. Generally, the size range of interest in nanotechnology is from 100 nm down to 1 nm, although in pharmaceutical nanotechnology the higher size limit is considered to be at 1000 nm [14]. Within this size range, materials can have properties that significantly differ from those expected for the same material at a larger size. These properties emerge from the nano-scale and are of great interest for some applications. By now, a plenty of nanomaterials is available on the market, applied in different products, like food, medicines and computer chips.

When nanotechnology is used for diagnosis or treatment of human diseases, it is called nanomedicine [13]. In this field, the advances reached through pharmaceutical nanotechnology are particularly interesting. Some important drawbacks presented by certain drugs can be solved with nanotechnology approaches. Thus, biocompatible nanomaterials are being increasingly investigated mainly because of their potential for improving pharma-cokinetic aspects of several classical drugs [15].

Different nanomaterials are available for medical purposes by now, such as liposomes, polymeric nanoparticles, solid lipid nanoparticles, micelles, superparamagnetic iron oxide nanoparticles, and others [13]. One of the most exciting properties of these materials is their potential for delivering the drug at its target site. This feature makes the nanocarriers good candidates for delivering drugs at malignant tissues, thus minimizing side-effects related to the action of anticancer drugs in non-target tissues and increasing the efficacy of the therapy.

The first experiments on nanostructured drug delivery systems for anticancer therapy were performed in the early 1980's [16]. These experiments showed that these systems increased the efficiency of classical anticancer drugs [15, 17, 18]. It was shown that the increased efficiency was indeed associated to the "tumoritropic" property of the nanoparticles [19]. Even by the time of those first experiments, it was known that tumor tissues allow for the passive accumulation of particles within a certain size range [19].

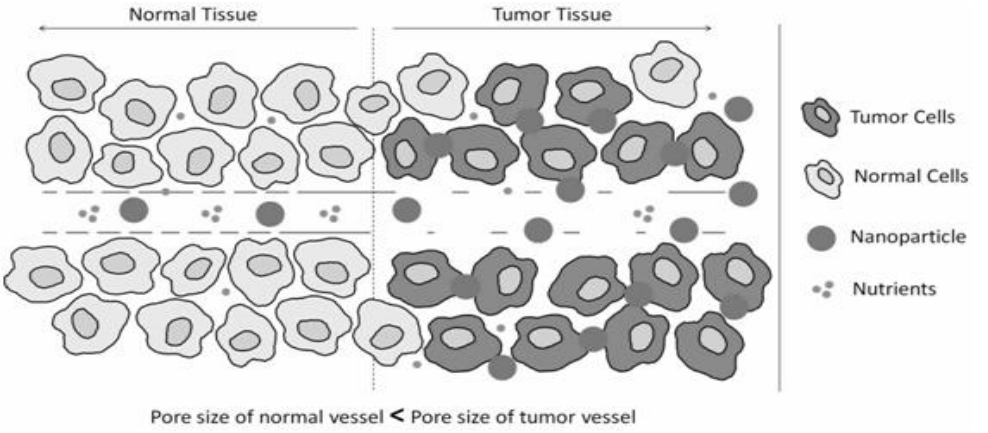

Figure 4. Representation of the enhanced permeability and retention (EPR) effect in a tumor. EPR is due to high microvascular permeability and poor lymphatic drainage observed in malignant tumors. The endothelial lining is represented by discontinuous horizontal lines.

The passive accumulation of nanostructures in solid malignant tissues is possible because tumors have an abnormal vasculature. The blood vessels in malignant tissues present a endothelial lining with larger pores than in normal vessels [13].

Therefore, the tumor microvascular permeability is generally higher than that expected for normal tissues [20] and it means that some nanostructures could penetrate in malignant tissue more intensely than in normal tissues. Indeed, experiments have shown that relatively large nanostructures can reach the tumor interstitium. In some tumors, the vascular permeability is high enough that allows the penetration of liposomes of 400 nm in diameter [20]. Besides the high microvascular permeability, malignant tumors show poor lymphatic drainage, which contributes to the accumulation of nanoparticles in tumor tissue [21]. Both high microvascular permeability and poor lymphatic drainage in malignant tumors are responsible for the enhanced permeation and retention EPR effect observed in this kind of tissue (Figure 4). Thus, nanostructured drug delivery systems could be design to passively accumulate in malignant tumors. Theoretically, it would be just a matter of controlling their size – actually, it is only a part of the truth, the interactions between nanostructures and the biological systems make it a little more complicated –. In general, nanostructures of about 100 nm in diameter are considered to be effective in penetrate and to be retained by tumor tissues [22, 23].

The surface of nanostructured drug delivery systems can also be modified in order to increase their affinity for the tumor tissue [21]. This strategy is named active targeting and is explored by several researchers in the development of various anticancer formulations nowadays [21]. Actively targeted nanosctructured drug delivery systems can be obtained by attaching targeting molecules to the surface of the drug-loaded nanostructures. Molecules such as antibodies, cationic peptides and folic acid can easily be conjugated to the surface of these systems and several conjugation protocols can be found in the literature [13, 24]. The active targeting can work together with the passive accumulation of nanostructures in malignant tumors, potentially resulting in significant gains in efficiency and safety in the anticancer therapy.

Nanostructures can also be used for other purposes, such as to improve the compatibility of hydrophobic drugs with aqueous media, to promote a sustained release of a drug in a

certain site of the body and to modify the drug circulation half-life in blood [13]. Particularly, as discussed in the following sessions, as most of the PS molecules are highly hydrophobic, nanostructures are holding interest in the field of PDT not only for acting as good drug delivery systems, but also because of their potential for increasing the compatibility of hydrophobic molecules with aqueous media.

4. Nanostructured Third Generation Photosensitizers for Anticancer Therapy

The development of nanostructured third generation PS for PDT is a relatively recent activity. It began about 20 years ago [13]. However, the number of published works involving nanoparticles and PDT has exponentially grown in the last five years [25]. Among the available nanostructured platforms, the liposome- [26] and biodegradable polymer-based [27] ones are the most often used for the development of third generation PS. These two important nanoplatforms are discussed in the sequence.

4.1. Liposomes

Liposome is the generic term used for lipid vesicles ranging in size from 20 to 1000 nm and consisting of one or more concentric phospholipid bilayer surrounding an aqueous media [13, 26]. An interesting feature of liposomes is their amphiphilic nature, which allows for the association of both hydrophobic and hydrophilic PS molecules to its structure. Hydrophilic molecules can be entrapped in its aqueous core, while the hydrophobic ones can intercalate the lipids of the liposome membrane. Other attractive feature of liposomes is the readiness for tuning their physical properties by controlling their lipid composition and by using simple formulation methods.

The phospholipids used in liposome formulations can be synthetic or derived from natural sources [26]. The main characteristic of these lipids is the presence of a polar head attached to long hydrophobic carbon chains through a glyceryl backbone. This feature along with the shape of the phospholipids is an important determinant for the shape and size of liposomes [26]. Moreover, the surface properties and the charge of liposomes can be controlled by modifying the formulation composition. Other lipids, such as cholesterol, can be added to the liposome formulation in order to modify its characteristics and improve its stability [26]. Generally, for prolonging the circulation half-life of liposomal drug-carriers in blood, functionalized lipids are added to the formulation. The most used stabilizer is polyethylene glycol (PEG). This is necessary because plain liposomes are rapidly phagocytosed by macrophages of the reticulo-endothelial system (RES) after their intravenous administration [13, 26].

So far, there is only one liposomal PS approved for clinical use, but it is not indicated for anticancer PDT. This formulation is known as Visudyne®, a third generation PS consisting of a liposomal formulation of verteporfin used for PDT against age-related macular degeneration [13, 26].

In terms of preclinical tests, a well studied liposomal formulation of PS is the 5,10,15,20-tetrakis(m-hydroxyphenyl)chlorin (m-THPC, Foscan®) loaded into dipalmitoyl phosphatidyl-choline/dipalmitoyl phosphatidylglycerol (DPPC/DPPG) liposomes, also known as Foslip®. Several studies show that Foslip® presents improved characteristics, such as higher efficiency, enhanced selectivity and reduced side-effects, in comparison to the non-liposomal formulation, Foscan® [13].

Liposomal formulations of phthalocyanines are also under study. The molecules of the phthalocyanine family show photophysical and photochemical characteristics that are very attractive for PDT protocols. Among these characteristics, it could be mentioned that they strongly absorb light at wavelengths near 700 nm, have a very stable triplet excited state, are retained for long times by cancer cells, and are very photostable [9, 10, 28, 29]. However, the classical phthalocyanines are highly hydrophobic, a feature that make them aggregate when in aqueous media and to present side-effects/poor efficiency related to accumulation in non-target tissues [29]. Chemical modifications in some phthalocyanines were made in an attempting to improve their characteristics, but the hydrophilic derivatives, such as sulphonated phthalocyanines, lack several interesting properties presented by the hydrophobic ones [29]. The solution for these problems could be a formulation that maintain the good characteristics of hydrophobic phthalocyanines and improve their pharmacokinetics for anticancer PDT. In this context, liposomal phthalocyanines formulations promise to be interesting third generation PS for anticancer PDT and are already under study [30].

Our research group has investigated the efficacy of aluminum-phthalocyanine-loaded liposomes (AlPcCl-LP) against several superficial cancer cells, both *in vivo* and *in vitro*. AlPcCl-LP showed high efficacy against KB cells (human nasopharynx carcinoma cells) kept in culture; the reduction in cellular viability reached 100 % after AlPcCl-LP-based PDT and it involved the induction of both necrosis and apoptosis [31]. *In vivo* experiments using small animal tumor models showed that AlPcCl-LP was effective against tongue tumors (Ehrlich tumor cells); 24 h after PDT application, necrosis was induced in 90 % of the tumor tissue [32]. In addition, the AlClPc-LP was also effective against primary canine breast cancer cells (results to be published soon).

Liposomes also allow for the conjugation of targeting molecules to their surface. The targeting molecules can be attached to the liposomal membrane either before or after formation of the vesicles [13]. This strategy is based on the fact that some cancer cells overexpress some cancer-associated molecules, such as receptors for vitamins (folic acid), receptors for glycoproteins (transferrin) or certain surface antigens [13, 26]. The resulting liposome can then be actively targeted to tumor tissue. It was reported that by conjugating transferrin to liposomes loaded with a sulphonated phthalocyanine (AlPcS4), the uptake of the photosensitizer by cervical cancer cells was significantly increased [33].

4.2. Biodegradable Polymeric Nanoparticles

Polymeric nanoparticles present characteristics that make them good candidates for PS-delivery systems for anticancer PDT. They can be considered as an alternative to liposomes when liposomal formulations do not present the desired characteristics. Some polymeric nanoparticles allow for the control of drug release [24], the maintenance of photodynamic activity of hydrophobic PS in aqueous media [34, 35], the loading of high drug concentrations

[24], and the easy chemical modification of their surface [24]. Much effort has been applied on the search for biodegradable polymeric nanoparticles-based drug delivery systems. Particularly, the systems based on poly(lactic, glycolic acid) (PLGA) have been intensely studied [13, 24].

Konan and cols. (2003) obtained PLGA nanoparticles loaded with the PS meso-tetra(4-hydroxyphenyl)porphyrin (p-THPP) and smaller than 130 nm in diameter [23]. This diameter, as discussed before, enables the nanoparticles to passively accumulate in malignant tumors. These nanoparticles were obtained through an emulsification-diffusion process, which yielded interesting results: a drug loading of about 8 % (m:m) and a PS entrapment efficiency up to 77 %. The polymer itself was not toxic to EMT-6 cells (mouse mammary cancer cells) kept in culture, as expected. However, the final system composed by p-THPP entrapped in PLGA (50:50) showed a high photodynamic activity against EMT-6 cells. When compared to the free p-THPP, this nanostructured drug delivery system presented a significantly higher photocitotoxicity *in vitro* [23]. The authors suggested that the PLGA nanoparticles could deliver the PS to specific intracellular compartments, not accessible to the free PS, where it would be more effective in inducing cell death. In another work, zinc(II)-phthalocyanine was encapsulated in PLGA nanoparticles with an efficiency of 70 % through the method of solvent emulsion evaporation [36]. The obtained nanoparticles were spherical-shaped, with a smooth surface, presented a mean hydrodynamic diameter of 285 nm and maintained the expected photophysical properties of zinc(II)-phthalocyanine. This drug delivery system showed a slow release of the PS in *in vitro* conditions. In the first 3 days, about 15 % of the zinc(II)-phthalocyanine were released. After this burst, the PS was slowly released, reaching only 40 % of the total drug load after 25 days. This sustained release is typical for PLGA nanostructured systems [36]. As expected, the phthalocyanine-loaded PLGA nanoparticles were not toxic in the dark, but presented intense phototoxicity, to cultured cells – the authors tested it in a murine macrophage cell line (P388-D1) –. These results showed once again that PLGA nanoparticles are suitable as nanocarriers for PS.

Conclusion

Anticancer PDT is not only a promising therapeutic alternative for the future; it is in fact a choice available for the treatment of several cancer types nowadays. However, some drawbacks, mainly related to the non-specific localization of PS molecules in the body, still represent a real limit to a wide clinical use of anticancer PDT. Thus, improvements on PS deliver to cancer cells have been a hot issue in current researches. Investigations on PS-delivery systems led to the development of the so-called third generation PS. In this context, nanotechnology has recently emerged as a rich source of useful innovations in the field of drug delivery systems. Through the use of nanosized PS carriers, researchers have shown that it is possible to specifically target cancer cells, increasing both efficiency and safety of this anticancer protocol. Several nanostructured PS-delivery systems are described in the literature but more studies are necessary in order to make them available for clinical use.

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Chapter 3

The Role of Phagocytosis in Cell Deaths by Photodynamic Therapy

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Abstract

Resistance to cell death and the ability to overrule immunosurveillance represent two hallmarks of tumour cells. The optimal cancer treatment should combine the induction of cell death and immunostimulatory effects based on immune cells activation consequent to the immunogenic characteristics of dead cells and their removal.

The dynamic interplay between dead cell and phagocytes leading to efficient phagocytosis is divided in four steps: 1) attraction and accumulation of phagocytes to the site of apoptotic cells, 2) recognition and tethering, 3) internalization and 4) processing of dead cells within the phagocytes avoiding phlogosis. Apoptotic cells secrete chemotactic signals attracting phagocytes and expose on plasma membrane ligands mediating receptor-specific physical contact with phagocytes. Indeed, the subsequent internalization steps and outcomes, i.e. immunotolerance or immunomodulation, profoundly depend on the phagocytic receptors involved.

PhotoDynamic Therapy (PDT), a promising new cancer treatment, is based on the synergic action of photosensitizing drugs (PS) and light irradiation that allow to kill the cells respectively *via* chemical and physical stress. Highly Reactive Oxygen Species (ROS), formed by the transfer of energy absorbed by PS upon irradiation to molecular oxygen, mediate the destruction of target cells. The best feature of PDT is the ROS damage directionality, depending on PS localization that in turn is mediated by its hydrophilicity/hydrophobicity, and the precise delivery of light to the treated sites. Multiple advantages characterize PDT as cancer treatment modality and make it potentially capable to meet many currently unmet medical needs. Particularly, PDT elicits a strong acute inflammation, orchestrated by both the innate and adaptive immune

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system, ensuring a protective effect by containing the disruption of tissue homeostasis and removal of damaged cells. The acute inflammatory response depends on photosensitized dead cells. Indeed, PDT not only induces apoptosis, autophagy and necrosis in the tumour cells, but it is also effective to rapidly generate an abundance of alarm/danger signals, called Damage-Associated Molecular Patterns (DAMPs), detected by the innate immunity alert elements. These, recruited to the sites of dead cells, eliminate injured and dead cells by phagocytosis and trigger the antitumour immunity by maturation and activation of dendritic cells (DCs).

Here we will discuss whether the multiple PDT-induced cell death types can result in an immune response, linked to the exposure and/or release of signals by dying cells, ending in the phagocytosis by the immune cells.

Introduction

PhotoDynamic Therapy (PDT) is a Food and Drug Administration (FDA)-approved modality for both the treatment of early-stage cancerous and noncancerous diseases and palliation of late-stage ones [1]. PDT consists of three basic individually non toxic components: photosensitizer (PS), light and oxygen [2, 3] that in sinergy trigger a photochemical reaction culminating in the generation of highly Reactive Oxygen Species (ROS).

PDT utilizes the destructive power of ROS generated *via* visible light irradiation of the PS accumulating in the cancerous tissue/cells, to elicit their obliteration. This result is gained by multifactorial effects: (1) direct tumour cell kill, (2) damage to the vasculature, (3) rapid recruitment and activation of immune cells that can facilitate development of anti-tumour adaptive immunity [4-6]. These effects strictly depend on determination of photodynamic treatment optimal conditions, i.e., PS type and dose, light total dose and fluence rate, tumour oxygen concentration. PDT has been shown to reduce the number of clonogenic tumour cells by efficiently inducing the three main cell death types, i.e., apoptosis, autophagy and necrosis [4, 7].

The massive presence of dead photosensitized cancer cells may provide the immunogenic *impetus*, achieved by a series of intracellular molecules, categorized as Damage-Associated Molecular Patterns (DAMPs) and normally hidden within live cells, extracellularly secreted or exposed on plasma membrane of dying cells. This implies the phagocytosis of dead cells, Dendritic Cells (DCs) activation and maturation, antigen processing and T cell activation [8]. The failure of these processes causes the onset of autoimmune disorders, such as Lupus Systemic Erythematosus (LSE), cystic fibrosis, Chronic Obstructive Pulmonary Disease (COPD) [9-12], that along with the neoplastic disease impairs clinical conditions of the patient. Here we summarize the most important findings about the immunogenicity of the multiple PDT-induced cell death types.

Principles of PDT

PDT is a form of photochemotherapy, requiring a PS, the visible light and tissue oxygen. Technically, PDT combines two steps: the administration of a light-sensitive PS and irradiation with a light of appropriate wavelength matching the absorption *spectrum* of PS.

Photosensitizers

A PS is a molecule activated to an excited singlet state when it absorbs a light photon. In PDT, either a PS or its metabolic precursor is administered to the patient. The ideal PS should have: 1) easy synthesis; 2) little or no dark toxicity; 3) no mutagenicity and/or carcinogenicity; 4) good pharmacokinetic behaviour, i.e. high selectivity/targetability for tumour tissue and easy elimination from the patient; 5) reliable activation by an appropriate wavelength; 6) versatile administration; 7) commercially available; 8) low cost; 9) safety; 10) red absorption for deeper tissue penetration; integrative ability with other clinical treatment, e.g. surgery, radiation and chemotherapy [13].

PSs classification includes a multitude of schemes, all with limitations. However, PSs can be generally categorized by: a) generation; b) synthetic purity; c) targeting and d) chemical structure [14].

a) Generation. Depending on when they were generated, the PSs can be divided in three generation groups: the first comprises Hematoporphyrin and its derivatives, signed HpD; the second contains expanded porphyrins, chlorophyll derivatives and dyes; the third covers 1st and 2nd generation PSs conjugated to biological modifiers, e.g. antibodies and nanoparticles [15, 16].
b) Synthetic purity. Although the PSs composed of multiple components are chemically impure, they do not result clinically inferior. In this context, despite its multiple components, Photofrin, the most common PS for oncologic PDT, offers reproducible and reliable therapy.
c) Targeting. The PSs are classified on the basis of their intracellular specific localization, such as cell membranes, subcellular membranes or organelles. The smaller monomers HpD localize the mitochondria, while larger dimers and oligomers cross plasma membrane by phagocytosis [17]. Chlorin e6 (MACE) is carried to lysosomes by endocytosis [18, 19]. Pthalocyanines localize in mitochondria [20]. Benzoporphyrin derivatives (BpD) accumulate in the Golgi apparatus [21]. Aminolevulinic acid (ALA) crosses the cell membranes, lysosomes and mitochondria [22].
d) Chemical structure. Chemical structure-based PSs classification presents relevance for synthetic chemists, but has a limited clinical impact. The tetrapyrrole ring represents the basic frame of most PSs, which can be broadly classified as derivates of three main chemical compounds, i.e. porphyrins, chlorines and bacteriochlorines. Chemical alterations in the side chains groups may both improve PS activity and cause toxic side effects. Indeed, synthesis molecules deriving from chemical laboratory are rarely useful PSs for clinicians.

Since all attempts of PSs schemes present several limitations, the best way to classify clinical PSs probably relies on their action mechanism, which would make a PS specific for a particular neoplasia.

PSs chemical properties, i.e. net ionic charge, hydrophobicity degree and asymmetry degree of the molecule, lead their subcellular localization and, in turn, the cellular site and type damage. In general, hydrophobic PSs with two or less negative charges accumulate in plasma and intracellular membranes, while hydrophilic ones, as well as aggregated states of

PSs, are taken up by endocytosis and pinocytosis localizing in lysosomes and endosomes [18, 23]. Upon irradiation, the first induce lipids and proteins photooxidation triggering the activation of membrane phospholipases with consequent fast degradation of damaged phospholipids, changes in fluidity, rapid loss of cell integrity, enzyme and receptors systems inactivation and cell membrane depolarisation with ionic balance collapse. The second induce lysosomal enzymes release to the cytosol. PSs localizing in the cytosol can bind the tubulin and depolymerise microtubules with accumulation of cells in mitosis and subsequent cell death.

Light Sources

In PDT, the administration of the PS is followed by its activation using a light (typically visible or infrared) at a specific wavelength.

The interaction of light with biological tissues is very important in photodynamic treatment, since the photons crossing the tissue undergo several processes of absorption, reflection, refraction, scattering, transmittance and fluorescence exploited in medicine.

- Absorption: optical phenomenon, governed by Lambert-Beer's law, occurring when a photon carries an energy that matches the gap between two energy levels in a molecule. It is mainly due to the presence of endogenous tissue chromophores (e.g. haemoglobin, myoglobin, melanin and cytochromes) that define the "tissue optical window" as the wavelength range between 630 and 1300 nm where the light deeply penetrates.
- Reflection: optical phenomenon, governed by Fresnel's law, determined by re-emission of photon beam impinging onto tissue surface.
- Refraction: optical phenomenon, governed by Snell's law, observed when a photon beam penetrates the tissue undergoing a change in direction.
- Scattering: optical phenomenon, defined by Rayleigh-Gans theory, occurring when a photon beam penetrating the tissue is spread in multiple beams with different directions defined by the anisotropy factor.

Among all these optical phenomena, absorption and scattering play a key role in photodynamic treatment.

The prediction of light spatial distribution in the target photosensitized tissue is very important since the light can be absorbed or scattered depending on tissue type and geometry and light wavelength. Indeed, biological tissues are inhomogeneous because of the presence of macromolecules, cell organelles, organized cell structures, interstitial layers making them turbid. One of the most important limiting factors in PDT is the penetration depth of the light in the tissue during the treatment due mainly to scattering influencing light fluence rate in the underlying cell layers [24]. The product of the fluence rate and the treatment time define the total light dose given during PDT to obtain the optimal penetration depth. In addition, the PS itself can absorb the light, a phenomenon known as "self-shielding", limiting the tissue light penetration [25]. Light exposure can also photodestroy the PS, a process referred to as "photobleaching", reducing in turn photosensitisation efficiency [26].

In PDT several light sources can be used for the therapeutic irradiation. The choice depends on tumour properties (e.g. localization and geometry), light dose delivered and PS. The light sources available for PDT, divided in incoherent (e.g. a tungsten lamp) or coherent (e.g. laser) sources, belong to three main groups, i.e. broad spectrum lamps, diode lamps and lasers. Their wavelength correspond with the Soret band and four lower peaks of absorption, refereed to as Q bands, along the porphyrin curve. The Soret band (400-410 nm), with a maximal absorption at 405-409 nm, is the highest peak, while Q bands, at approximately 505-510, 540-545, 580-584 and 630-635 nm, are the smaller peaks. The majority of clinical studies uses light wavelengths ranging between 625 and 633 nm, allowing a deeper skin penetration [27]. The non-coherent light sources comprise halogen lamps, light-emitting diode (LED) lamps and intense pulsed light (IPL) [28]. They can be used with various PSs and are safe, user friendly and relately inexpensive. Moreover, these light sources can be used combined with optical fibers to assure selective wavelength. Their disadvantages include thermal effect, low light intensity and uncontrolled light dose. LED are an emerging PDT non coherent light source since they can generate high energy light at desired wavelength and can be assembled in a range of geometries and sizes. LED are directional light source with the maximum emitted power in the direction perpendicular to the emitting surface. In contrast to incoherent light sources, lasers provide high energy monochromatic light of a specific wavelength with a narrow bandwidth for a specific PS. Lasers present focal precision allowing small lesion treatment with minimal damage to the surrounding tissue and within a short time interval. However, incoherent light sources irradiation is more reliable, simpler, and cheaper and usually shows similar efficacies as compared to laser irradiation [29, 30].

Therefore, incoherent light sources represent the gold standard in topical PDT using protoporphyrin-based PSs [31, 32].

Tissue Oxygen

While sufficient quantities of PS and light dose are exogenous prerequisites for the photodynamic effect, the production of the PDT-induced cytotoxic mediators directly depends on the endogenous presence of the tissue ground-state molecular oxygen. Indeed, the efficiency of the photosensitisation is a direct consequence of the yield of the singlet oxygen ($^1O_2$), the most reactive oxygen form species mediating PDT cytotoxicity, in the tumour environment. The yield of $^1O_2$ in turn depends on the *in vivo* concentration of oxygen in the tissue. Hypoxic cells are very tolerant to photosensitisation and the photodynamic reaction mechanism may itself consume oxygen at a rate conducive to induce a state of temporary hypoxia and stifle further photosensitisation effects. On the contrary, it has been reported, that hyperbaric oxygen can improve the effect of photosensitization [33].

Photophysics and Photochemistry of PDT

The basis of PDT is the induction, in the target tumoural tissues, of toxic photodynamic reactions leading to ROS generation by photochemical and photophysical processes. In the dark, PSs exist in the ground state (S_0) with paired electrons; upon absorption a light photon, one of these electrons is shifted to a previously unoccupied orbital of higher energy and

inverts its spin forming the extremely unstable singlet excited state, S_1, with a half life ranging between 10^{-6} and 10^{-9} seconds. The singlet excited PS either decays back to the ground state, resulting in the fluorescence or undergoes intersystem crossing to the longer lived (10^{-3} second) triplet excited state (T_1), where two electrons are unpaired and have the same spin. The rate-limiting factor in the success of the photodynamic reaction is the ability of the PS to get excited to the higher, long-lived energy rich triplet state upon irradiation. Most PSs have a high *quantum* efficiency for S_1-T_1 transition. Activated PSs in the metastable triplet state may energize photochemical reactions inducing chemical changes in the surrounding molecules *via* two competing types of photo-oxidative reactions. Type-I photochemical reaction involves the transfer of electrons (or protons) from the PS to water or biomolecules to form a radical anion or cation, respectively. These radicals may react with molecular oxygen to produce peroxides, superoxide ions and hydroxyl radicals, initiating free radical chain reactions. In type-II photochemical reaction, the triplet excited state PS mediates the energy transfer process with molecular oxygen, returning to its ground state S_0. The type II reaction results in the formation of a highly toxic activated oxygen molecule, singlet oxygen ($^1O_2$), with very short life time (< 40 nsec) and action *radius* (< 0.02 μm). The *in situ* generation of $^1O_2$ plays a key role in photodynamic cytotoxicity because of its efficient interaction with a large variety of biomolecules, such as proteins, nucleic acids and lipids. Type II mechanism dominates in PDT, but both type I and II reactions occur simultaneously and the *ratio* depends on several parameters, including the PS used and oxygen concentration [34].

Cell Death Mechanisms after PDT

In PDT, photochemical reactions induced by optical activation of the PS molecules in the target tissue lead cancer cells demise through multiple pathways. Cell death, initiated by intracellular generation of singlet oxygen and other ROS, results from direct cellular damage, vascular shutdown and, only in recent years, from a PDT-induced activation of the immune response [4-6]. Tumour cells can respond to the direct photodynamic injury by turning on specific genetically programmed cell death (PCD) pathways, including apoptosis (type I PCD), autophagy (type II PCD) and necrosis (type III PCD). However, the direct photodamage is not thought to be sufficient for cancer resolution. To this purpose, PDT-induced vascular collapse plays an important role contributing to hypoxia and nutrient deficiency. Indeed, the viability of neoplastic cells also depends on the amount of nutrients supplied by the blood vessels, which in turn hinge on growth factors produced by tumour or host cells.

The mechanisms underlying the vascular effects of PDT differ greatly with PSs, but vascular constriction, thrombus formation and inhibition of tumour growth are common to the most important ones [35]. In addition, PDT can induce strong inflammatory and immune reactions that mediate tumour destruction by the rapid recruitment of immune cells, such as macrophages (Mφ), DCs and granulocytes, to the sites of neoplasia. In fact, several studies report the infiltration of lymphocytes, leukocytes and macrophages into PDT-treated tissue producing an activation of the immune response that consequently eliminates surviving cancer cells escaped to the direct PDT effects [8, 36-38].

PDT and Apoptosis

PDT can directly kill cancer cells by the efficient induction of apoptosis both in cultured cells and *in vivo*. Different cells types are committed to apoptosis after PDT with many PSs and evidences of apoptosis are reported in several tumour biopsies [39-41]. The ability of the photosensitized cells to initiate the apoptotic machinery depends mainly on the cell line, the overall light dose, the type and concentration of the PSs [42-46]. Particularly, the onset of apoptosis relies on the cellular photodamage sites induced by the specific PS localization. Mitochondria, lysosomes, plasma membrane, nuclei of tumour cells and tumour vasculature have been evaluated as potential PDT targets. PSs localizing in mitochondria, including porphyrogenic PSs and phthalocyanine-related compounds, activate the apoptotic pathway within a precise threshold of oxidative stress, which is dependent on the light dose and intracellular PS concentration. PSs targeting the Endoplasmic Reticulum (ER) damage calcium pumps, stimulating a rapid increase in the intracellular calcium concentrations ($[Ca^{2+}]_{cyt}$) immediately after photoactivation. Lysosomes-associated PSs have been shown to harm to lysosomal membrane, resulting in the release of cathepsins and other hydrolytic enzymes that can activate mitochondrial apoptosis *via* Bid [47].

Apoptosis, or Type I PCD, is a highly regulated cell suicide program in which several signaling cascades trigger a series of morphological and biochemical changes leading to cell demise. Misregulation of apoptosis has been implicated in a number of diseases, particularly massive apoptosis of neurons consequent to toxic protein aggregates and oxidative stress contributes to Alzheimer and Parkinson's pathologies, while failure in apoptosis execution causes atherosclerosis, chronic inflammation and cancer [48, 49].

Apoptosis consists of 4 steps: (1) the decision to activate the pathway; (2) the actually "suicide" of the cell; (3) engulfment of the remained cell by specialized immune cells called phagocytes; (4) degradation of engulfed cell.

Apoptotic cell death is primarily defined by a pattern of morphological hallmarks involving chromatin condensation, cleavage of chromosomal DNA into internucleosomal fragments, cell shrinkage, plasma membrane blebbing and final disintegration of the cell into membrane-surrounded apoptotic bodies [50, 51]. These changes, readily observed *in vitro*, are rarely seen *in vivo*, since dying cells are efficiently recognized, engulfed and processed by phagocytic cells, e.g. macrophages, immature DCs, endothelial cells or fibroblasts, before they enter the late stages of the apoptotic process [52].

At the biochemical level, apoptosis entails the sequential activation of initiator and effector caspases, a family of evolutionarily conserved cysteine-dependent aspartate-specific proteases, functioning in both cell disassembly (effectors) and in initiating this disassembly in response to proapoptotic signals (initiators) [53-55]. Caspases are synthesized as inactive precursors, referred to as procaspases, and are activated by a proteolytic processing generating the tetrameric active caspase enzyme, composed of two repeating heterotypic subunits. While effector caspases only require a specific intrachain cleavage, the initiator ones rely on adaptor protein complexes – the apoptosomes. In most PDT-induced apoptotic programs, caspases activation seems to be sufficient, but not necessary for the execution phase. In fact, caspase-independent pathways can be involved in photosensitized cells demise. For example, Rose Bengal Acetate (RBAc)-PDT is able to induce apoptosis in HeLa cells through both caspase-dependent and caspase-independent pathways. Moreover, RBAc-PDT induces the independent activation of multiple apoptotic pathways [7, 56].

Apoptosis primarily proceeds *via* 'extrinsic' (death receptor activation) and 'intrinsic' (mitochondrial) pathways, in which initiator caspases-8, -10 and -9 directly activate the effector procaspases-3 and -7 [57]. The 'extrinsic' pathway is initiated when a cell surface death receptor (e.g., Fas, TNF-RI, TRAIL receptor) is activated by the binding of a specific extracellular death ligand (e.g., FasL, TNF-α, TRAIL). The consequent death receptors clustering induces the formation of the Death-Inducing Signaling Complex (DISC), which recruits the initiator pro-caspases-8 and -10 resulting in their dimerization-induced activation that in turn leads to the proteolysis of the main effector caspases-3 and -7 [58]. Pc 4 mediated PDT induces apoptosis in A431 cells *via* the cell surface receptor Fas pathway, resulting in a multimerization of Fas protein that leads to its activation [59]. Fas/FasL system also sustains apoptotic program in CNE2 and TW0-1 cells photosensitized with Hypocrellin A and B [60].

The mitochondrion is the central processing organelle for the intrinsic apoptotic pathway. Apoptosis, which is usually, but not exclusively, associated with caspase activation [61, 62] converges on mitochondria to induce Mitochondrial Membrane Permeabilization (MMP), responsible of the release into the cytosol of several apoptogenic proteins stored in the inter-mitochondrial membrane space [63, 64]. Among these, the cytochrome c acts as caspases activator since, by binding to Apaf-1 (Apoptotic protease-activating factor 1) and in the presence of ATP and dATP, it enables the activation of procaspase-9 through the formation of the heptameric complex, apoptosome [65, 66]. In the mechanism of cytochrome c release, a decisive role is played by the mitochondrial Permeability Transition Pore (PTP), a voltage-dependent, high conductance channel that requires a permissive load of matrix Ca^{2+} for the opening, formed at the contact sites between the inner and the outer mitochondrial membranes. PTP is a multi-protein complex composed by various soluble and membrane proteins, including the Voltage-Dependent Anion Channel (VDAC), the Adenine Nucleotide Translocase (ANT), Peripheral Benzodiazepine Receptor (PBR), Creatine Kinase (CK), HexoKinase (HK) and/or Cyclophilin D (CypD) have been found [67, 68]. However, the composition of PTP remains controversial and still a matter of debate. A long lasting PTP opening implies the dissipation of the proton gradient over the Inner Mitochondrial Membrane (IMM) with consequent loss of the mitochondrial transmembrane potential ($\Delta\psi_m$) and osmotic matrix swelling resulting in the breakdown of the Outer Mitochondrial Membrane (OMM). This rupture leads to the release of mitochondrial apoptogenic factors and to a drastic drop in the adenosine triphosphate (ATP) levels, triggering cell demise. In Pc 4 treated LY-R cells, the release of cytochrome c from mitochondria is not related to the loss of $\Delta\psi_m$, which, depending on PDT dose and post treatment time, occurs only in cells treated with the highest dose of the PS [69]. Similarly, cytochrome c translocation from the mitochondria into the cytosol is induced without any changes in transmembrane potential in HeLa cells photosensitized with Verteporfin [70]. Contradictory results have been obtained by Kessel and Luo, showing that the porphycenes-mediated PDT of leukemia P388 cells results in cytochrome c release concurrent with dissipation of $\Delta\psi_m$ immediately after photodynamic treatment [71].

Besides the PTP formation, an alterative mechanism proposed for MMP involves the Bcl-2 proteins, critical death regulators. The BCL-2 family of proteins includes both pro- (e.g. Bax, Bak, Bid, Bad) as well as anti-apoptotic (e.g. Bcl-2, Bcl-X_L, Bcl-w) members and the *ratio* between these two subsets contributes to arbiter cell survival [72]. Bcl-2 members are able to form homo- and hetero-dimers, suggesting neutralizing competition between them and to become integral membrane proteins. Particularly, the apoptotic *stimulus* induces the

insertion of cytosolic Bax into the OMM [73], where it forms, alone or along with other pro-apoptotic members, such as Bak or tBid (truncated Bid), supramolecular openings [74], resulting from the formation of homo-oligomeric Bax-containing pores or from the destabilization of the lipid bilayer. The molecular openings induced by Bax/Bak and/or Bax/tBid mediate the release of pro-apoptotic proteins (such as cytochrome c, Smac/Diablo and Apoptosis-Inducing Factor, AIF) from the intermembrane mitochondrial space into the cytosol [75-77]. In HeLa cells, by opening transition pore complex on the OMM, Bcl-2 pro-apoptotic proteins promote the loss of mitochondrial membrane potential ($\Delta\psi m$) and the consequent release, into the cytosol, of cytochrome c, ending in the cleavage cascade of caspase 9 and 3 [7].

Bcl-2 and other anti-apoptotic members associate with OMM and ER/nuclear membrane, where they inhibit the release of cytochrome c and the subconsequent pro-caspase 9 activation [78].

Photodynamic treatment is thought both to activate the pro-apoptotic members of the family and to photodamage Bcl-2 and other pro-survival molecules [79]. Indeed, 5-aminolevulinic (5-ALA) PDT induces a significant decrease in Bcl-2 mRNA expression and increased levels of Bax mRNA in cervical cancer cell line [80] and esophageal cancer cells [81]. Likewise human breast adenocarcinoma cells treated with Hypericin display Bcl- X_L downregulation and Bax upregulation [82]. The anti-apoptotic protein Bcl-2 is highly sensitive to photodynamic damage [83]: it can be either a direct target of ROS, responsible of specific Bcl-2 post-translational modifications post PDT or a downstream effector of transduction cascades initiated by the initial photodamage to other molecules. Presumably the dramatic Bcl-2 photo-oxidation with loss of its function represents a permissive signal for Bax/Bak-mediated MMP, whose molecular mechanism remains speculative in PDT.

Caspase 12 dependent pathway appears to be triggered by various stimuli that activate ER stress [84]. Caspase 12 is localized at the cytoplasmic face of the ER and is cleaved by the Ca2+-dependent protease m-calpain. Once cleaved, caspase 12 actives caspase-9 without formation of apoptosome [85, 86] or may interact with pro-apoptotic protein Bap31, a 28 kDa integral ER membrane protein containing a cytoplasmic domain that preferentially associates with caspase-8 [87].

A caspase 12-dependent pathway is also induced in HeLa cells after RBAc-PDT. Caspase 12-dependent apoptosis is triggered by ER stress, whose onset is proved by two ER stress sensors, i.e. eIF2α (eukaryotic initiation factor 2 alpha) phosphorylation and GRP78 (glucose regulated protein 78) up-regulation [7]. ER stress acts as a critical control point in several apoptotic pathways activated by stimuli causing Ca2+ overload or its homeostasis perturbation [88].

PDT and Necrosis

Necrosis has long time been considered a kind of accidental and uncontrolled cell death mechanism, morphologically characterized by vacuolization of the cytoplasm, swelling and breakdown of the plasma membrane resulting in the release of the whole intracellular content. Despite the idea of necrosis as a passive or default way to die, recent evidences suggest that its occurrence and course might be tightly regulated as part of a signal transduction pathway [89]. Indeed, necrotically dying cells initiate pro-inflammatory signalling cascades by actively

spilling inflammatory cytokines and other immunomodulatory factors that lead to their recognition and engulfment by phagocytes and the subsequent immunological response.

Promising therapeutic approaches reported to be able to induce necrotic death in cancer cells include PDT. PDT can induce necrosis at several levels [90, 91]. PSs localizing in the plasma membrane evoke necrotic cell death likely due to the loss of plasma membrane integrity with the consequent failure to maintain ion fluxes across it and rapid depletion of intracellular ATP, as reported in studies using Photofrin [92] or zinc(II) phthalocyanine [93]. Likewise, necrosis is the preferential cell death program induced by photosensitization compounds targeting lysosomes, since their activation results in the lysosomal membrane disruption and the release of lysosomal proteases mediating necrosis [94]. Accumulating evidence suggests that low doses of PSs induce cell death by apoptosis; conversely, PDT administrated at higher doses tends to switch cell demise toward necrosis. Overall, the initiation of apoptotic or necrotic cell deaths in response to the photodynamic insult rely on the crucial elements, including the cell type, light dose, PS concentration and subcellular localization, and oxygen partial pressure [4]. The study by Nagata and coworkers [95], employing the amphiphilic PS ATX-S10 (Na) and human malignant melanoma cells, demonstrated that light doses inducing less than 70% cytotoxicity cause mainly apoptosis, while features of necrosis were observed with doses leading to 99% cytotoxicity.

Other factors, such as intracellular Ca^{2+} overload and site of ROS generation, contribute to the onset of necrosis post-PDT. Particularly, Uzdensky and colleagues [96] reported the key role of Ca^{2+} and cAMP-mediated signaling pathways in photoinduced necrosis of isolated crayfish neuron and satellite glial cells treated with a mixture of different sulfonated aluminum phthalocyanines $AlPcS_n$. On the other hand, Pyropheophorbide-a methylester (PPME)-mediated PDT of colon cancer cells demonstrated that $^1O_2$ produced at the ER/Golgi membranes, the primary site of the photosensitization reaction, result in the activation of necrotic cell death, while other ROS than $^1O_2$ generated by mitochondria would promote the apoptotic pathway [46].

In HeLa cells RBAc photosensitization induces negligible necrosis only due to the absence of apoptotic cells clearance by phagocytes [7].

PDT and Autophagy

Autophagy is a self degradative process for the removal and turnover of damaged organelles and misfolded or aggregated proteins *via* the endosomal-lysosomal system [97]. Whereas apoptosis and necrosis irreversibly lead to cell death, autophagy can clear cells from stress factors and thus facilitate cellular survival by promoting catabolic reactions meeting the bioenergetic needs of cells. Autophagy involves the sequestration of cellular material within double-membrane bounded vacuoles, termed autophagosomes, and its subsequent degradation upon fusion of the autophagosomes with lysosomes [98].

Although autophagy is generally thought as a cell survival strategy, it has also been linked to programmed cell death triggered by the metabolic and bioenergetic collapse. Indeed, persistent autophagy, depleting the cell of organelles and critical proteins, can lead to a caspase independent form of cell death [99]. Reportedly, in some settings, autophagy acts as a molecular backup mechanism to execute cell death when apoptosis is inhibited [7]. The autophagic response to PDT is not fully elucidated, but several reports suggest that autophagy

plays a role in the photodynamic process, affecting the therapeutic outcome [100, 101]. In PDT paradigm, ROS represent the major source of cytotoxicity mediating oxidative stress and progressive reduction of cellular detoxifying and antioxidant enzymes or agents. Consistent with this concept, PDT may stimulate autophagy in order to solve the oxidative injury in a cytoprotective manner or promote autophagic cell death whether ROS-mediated damage affects the entire cellular machinery [102]. Therefore, autophagy, along with apoptosis and necrosis, contributes to the cytotoxicity of PDT protocols. Particularly, autophagy can occur independently of apoptosis in PDT treated cells and it serves as a protective mechanism in apoptosis competent cells, and it leads to cell death in cells that are apoptosis-deficient. Pc 4-PDT induced cell death in cells lacking procaspase-3 [103] or Bax [104] was neither necrotic nor apoptotic. Hypericin-based PDT of $Bax^{-/-}Bak^{-/-}$ double-knockout (DKO) murine embryonic fibroblasts (MEFs) protects from apoptosis, but kills the cells through a caspase-independent, autophagic cell death pathway [105].

Some proteins regulating the autophagic process are targets of photogenerated ROS. Among these, the ER-associated inositol trisphosphate (IP3) receptor, a key regulator of autophagy [106], is photodamaged by phthalocyanine PS Pc 4 [107]. Moreover, AlPcS(2a)-PDT targets the mammalian target of rapamycin (mTOR), a central regulator of cell growth and proliferation involved in the autophagic signaling pathway [108]. Conversely, other proteins participating in the autophagic process, such as Beclin 1, Atg5 and Atg7, do not present any relevant photodamage. Autophagy is induced in PDT protocols employing PSs that preferentially accumulate in late endosomes/ lysosomes (i.e., NPe6), ER (i.e., hypericin, CPO), mitochondria (i.e., mTHPC, BPD), or ER and mitochondria (i.e., Pc 4). On the other hand, a failed autophagic response occurs in cells treated with lysosomal PSs since either no lysosomes can fuse with the formed autophagosomes in order to complete the autophagic process, or the lysosomal enzymatic contents result dysfunctional upon irradiation.

Dead Cells Removal as Exploitable Resource in Cancer Therapy

One of the main hallmarks of cancer cells is the resistance to cell death. Particularly, during their evolution to the malignant state, tumour cells evolve multiple ploys to limit or circumvent apoptosis including the up-regulation of antiapoptotic regulators (Bcl-2, Bcl-xL) or survival signals (Igf1/2), the downregulation of proapoptotic factors (Bax, Bim, Puma), or the short-circuiting of the extrinsic pathway [109].

Moreover, different types of cell death trigger opposite input for the immune system, inducing tolerance or initiating adaptive immune responses.

Cancer cells carry out their intrinsic ominous program by exploiting the host immune mechanisms and avoiding the meshes of the immune surveillance. The abilities to replicate in a chronically inflamed microenvironment, to evade immune recognition and to suppress immune reactivity are specific skills acquired by neoplastic cells to escape the innate and adaptive immune responses [110].

The knowledge of cancer immune hallmarks could be channeled to reverse the situation in favor of the immune system and, eventually, the patient, undertaking therapeutic regimens capable to stimulate the immune reactivity and counteract immune suppression.

Therefore, the strategy holding the highest therapeutic value in the clinical management of the neoplasia should combine the restoration of cell death, by increasing the susceptibility of cancer cells towards death, and the retrieval of the immune surveillance, enhancing the immunological recognition of tumour cells [111, 112]. This may be achieved by favoring cancer therapies that induce immunogenic cancer cell death and avoiding cancer regimens that mediate immunosuppressive side effects, such as myelosuppression or thymolysis.

In addition, the removal of dead cells should be also considered. In fact, only dead cells, but not viable ones, shed antigens that can be presented to T cells. Moreover, only dead cells are able to attract phagocytes, such as DCs or macrophages also functioning as Antigen Presenting Cells (APCs), scavenging them and, consequently, they present antigens to T cells. Finally, tolerance or adaptive responses strictly depend on the cell death modality during antigen presentation [113].

PDT may meet these expectations, since it is able to induce acute inflammation characterized by increased expression of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 [114], adhesion molecules *E*-selectin and ICAM-1, and the rapid accumulation of leukocytes into the treated tumour area [115]. PDT enhancement of anti-tumour immunity appears to involve the stimulation of DCs by dying tumour cells [116]. Indeed, the incubation of photosensitized tumour cells with immature DCs implies an enhanced DC maturation, activation and ability to stimulate T cell activation [117]. Particularly, DCs engulf portion of dying cells, incorporate tumour-derived antigenic peptides into major histocompatibility complex (MHC) molecules and present them to T cells, thus stimulating the generation of tumour-specific CD8+ T cells [118, 119].

It is still unclear in which terms cellular demise provokes an immune response against dying tumour cells or rather remains immunologically silent.

The clearance of the dying cells, known as phagocytosis, is the genetically programmed final step of apoptosis. This process play a key role in normal tissue turnover, embryological tissue remodeling, immune system development and represents an evolutionarily conserved process to suppress inflammation and regulate immune responses [120]. Phagocytosis of senescent cells was first described in the late 19th century by Elie Metchnikoff in tadpole fins. In the early 1980s, a link between apoptosis (the term coined by Willie et al. [121] to describe a form of PCD) and cell removal was demonstrated in mammals and nematodes [122, 123]. Many genes are involved in the engulfment of apoptotic cells highlighting the relevance of this process. Among the 14 genes identified in the nematode *Caenorabditis elegans* related to PCD (*ced* genes), at least 7 (*ced-1, ced-2, ced-5, ced-6, ced-7, ced-10* and *ced-12*) code for proteins required for the dead cells engulfment whose mechanism involves two major pathways. The first involves CED-2, CED-3 and CED-5 proteins (mammalian homologues CrkII, Dock180 and ELMO, respectively), the second comprises CED-1, CED-6, and CED-7 proteins (mammalian homologues CD91, GULP and ABCA1 respectively). CED-10 (RAC1 in mammalian) functionally links the two pathways [124].

The engulfment pathways encoded by these genes in *C. elegans* are not specific for cells undergoing apoptotic cell death, but they are also required for the clearance of necrotic cells [125]. *C. elegans* lacks an immune system and dead cells are removed by neighboring cells [126]. Conversely, in mammals the phagocytic process involves many cell types, including professional phagocytes, i.e. DCs and macrophages, and non-professional ones, e.g. hepatocytes [127], epithelial cells [128], endothelial cells [129], fibroblasts [130] or glomeular mesangial cells [131], interplaying to remove dead cells [132]. Professional

phagocytes family consists of heterogeneous subsets of cells with different functional characteristics indicating the complexity of phagocytosis and its relevance [133, 134].

Phagocytosis mechanism depends on the dead cell type, the cell death inducer, the stage of apoptosis, the type and state of differentiation of the phagocyte, and the surrounding microenvironment [135-137].

Engulfment mechanism of apoptotic cells has been well described, clearance of necrotic cells is less well understood, conversely autophagic cells removal is at infancy.

Mechanisms of Clearance of Apoptotic Cells

Interaction between apoptotic cells and phagocytes can be divided into five steps: recruitment, recognition and tethering, phagocyte signaling and engulfment, processing of the ingested cargo and its degradation and, finally, release of cytokines and other molecules by the phagocyte mediating the immunological removal of apoptotic cells *in vivo* [118] (Figure1).

Recruitment, recognition, tethering, phagocyte signaling and engulfment are precisely orchestrated by a variety of ligands on the apoptotic cell, receptors on the phagocyte and bridge molecules in the microenvironment. Receptor-ligand interactions, often referred to as the 'third synapse' [138], amplify the signaling between ligand and receptor [132]. This requires a redistribution of the ligands into patches on the dying cells plasma membrane.

The tethering and engulfment two-step process is very crucial for the cell clearance and plays a key role in protection mechanism against the uptake of viable cells. In fact, phagocytes constantly monitor cells displaying either "eat me" or "don't me" signals, which induce stimulatory or inhibitory phagocytosis effects respectively.

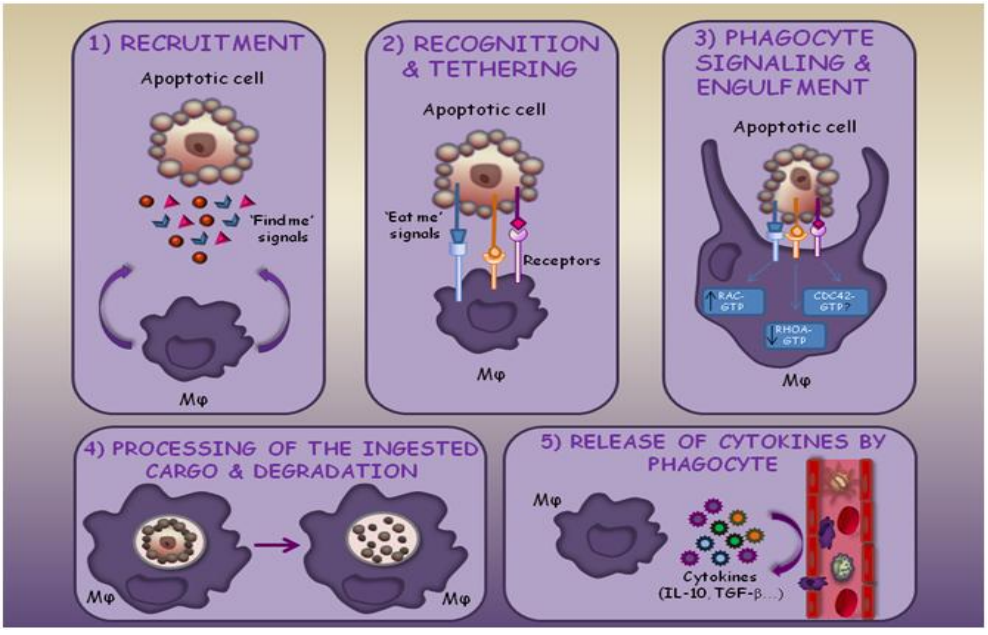

Figure 1. Schematic representation of the phagocytosis steps. Mφ: macrophage.

Particularly, the viable cells present molecules ("don't me" signals) promoting detachment from phagocytes or inhibiting phagocytic activity. These molecules are disabled when the cells undergo apoptosis and consequently expose "eat me" signals [139, 140]. Apoptotic cells are rapidly located by phagocytes owing to the release of soluble mediators, referred to as 'find me' signals, chemoattracting them [141]. Engulfment is stimulated by Rho GTPases Rac-1 and Cdc42 activation that affects cytoskeletal organization [142].

Stop the Recruitment and Recognition: Don't Eat Me Signals

Viable cells are not eliminated by phagocytes since they display on their plasma membrane molecules known as don't eat me signals. However, only few studies have been performed to understand their action mechanisms [140]. The well known don't eat me molecules include CD31, also called PECAM-1 (Platelet-Endothelial Cell Adhesion Molecule-1), [143] and CD47, also called Integrin-Associated Protein (IAP), [144] proteins. The inhibitory or stimulatory phagocytosis effect is based on the intracellular signaling mechanism induced during homophilic interaction between molecule expressed on viable or apoptotic cells and receptors displayed on macrophages. The intracellular mechanism to inhibit cell responses is mediated by phosphorylation of Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) contained into the receptors. ITIMs contain a six aminoacids *consensus* sequence (Ile/Val/Leu/Ser-X-Tyr-X-X-Leu/Val) [139].

Recruitment: Find Me Signals

In vivo, the apoptotic program is strictly linked to the prompt removal of dead cells, primarily based on soluble chemotactic factors released by apoptotic cells ensuring the recruitment of hordes of phagocytes [145]. In fact, several molecules have been shown to be capable of functioning as "find me " signals. These include lysophosphatidylcholine (LPC) [141], fractalkine [146], nucleotides ATP and uridine 5' triphosphate (UTP) [147], sphingosine 1-phosphate (S1P) [148, 149] and a fragment of the tyrosyltRNA synthetase (EMAPII) [150]. Moreover, also the cross-linked homodimer of S19 ribosomal protein can function as "find me" signal [151], as blebs derived from apoptotic germinal center B cells [152] and the bridging molecule thrombospondin derived from apoptotic cells [153].

All of these molecule are released when the apoptotic cells plasma membrane is still intact and are capable to attract the monocytes. Fractalkine and nucleotides function also *in vivo* as chemoattractant [146, 147]. Particularly, the "find me" concept is related to the chemotactic gradient range, strictly dependent on the tissue "find me" molecules concentration, degradation and modification and possibility to get out the tissues into the circulation.

MCF-7 breast cancer cells attract the THP-1 monocytic cell line after release of LPC based on caspase-3-mediated activation Ca^{2+}-independent phospholipase A2 (iPLA2) in apoptotic cells (Lauber et al., 2003). Since serum or plasma are characterized by a high concentration (100 μM) of LPC that could cause the lysis of many cells, probably LPC is not recognized in its native form but bound to other serum components and further modified in the tissues. Alternatively, LPC may function together with other soluble mediators. The

specific receptor on phagocytes mediating LPC-dependent movement towards apoptotic cells is unclear. Kabarowski and coworkers suggest that G2A can function as receptor linked to LPC in recognition by phagocytes [154].

Truman et colleagues [146] suggested that the apoptotic Burkitt Lymphoma cells induce monocytes migration both *in vitro* and *in vivo* by releasing a soluble fragment of chemokine fractalkine (CX3CL1), an intercellular adhesion molecule localized on plasma membrane. CX3CL1 is released as a 60 kDa fragment in a caspase dependent manner. Moreover, fractalkine can be released as part of microparticles from early stages apoptotic Burkitt Lymphoma cells. CX3CR1 is the receptor involved in sensing the chemokine and inducing migration as suggested in CX3CR1 deficient mice, defective in macrophages migration to the germinal centers.

The regulated release of triphosphate nucleotides ATP and UTP from early stage apoptotic primary thymocytes, Jurkat, MCF-7 and lung epithelial cells induces *in vitro* migration of monocytes. Their supernatants injected into the mice induce apoptotic cell clearance and the degradation of ATP and UTP abolish the recruitment. The sensing of the released extracellular nucleotides involves the P2Y family nucleotide receptors, whose signaling downstream inducing monocytes migration remains to be elucidated [147].

Nucleotide triphophatases (NTPases), expressed at different amounts in various tissues, degrade extracellular nucleotides and regulate the distance for the attraction signal [155, 156].

The sphingosine 1-phosphate (S1P) is another soluble molecule proposed as find me signal [148, 149]. Two mechanisms have been proposed to be linked to S1P generation. The first involves the upregulation of S1P kinase 1 (SphK1) induced in apoptotic cells [148], conversely the second is based on caspase 1-induced cleaved fragment of S1P kinase 2 (SphK2) released from dying cells into the extracellular space where it would generate S1P [149]. However, the involvement of S1P as a recruitment signal has not been determined *in vivo* and its generation mechanism also remains to be better defined. Moreover, although the G protein-coupled receptors (GPRSs) $S1P_1$ and $S1P_5$ on phagocytes have been proposed as sensing mechanism, its relevance for phagocytic recruitment to apoptotic cells is unknown.

Ribosomal protein S19 dimerizes during apoptosis and acquires the capacity to bind the membrane receptor C5a; consequently attracted monocytes phagocyte the very late stages apoptotic cells [151]. Endothelial-monocytes-activating polypeptide II (EMAPII) is a domain of p43 aminoacyl-tRNA synthetase complex component, released by apoptotic cells, able to induce migration of human mononuclear phagocytes [150]. EMAPII is an inflammatory cytokine also attracting and activating neutrophils.

In addition to create a chemotactic gradient towards professional phagocytes not located in the proximity of apoptotic cells, “find me” signals also modulate the phagocytic activity of non professional phagocytes neighboring dying cells as reported in apoptotic airway epithelial cells soon eaten by epithelial cells [147]. Moreover, in mammals “find me” signals could upregulate phagocytic machinery both in professional and non professional phagocytes [157], also influencing immunogenic or not responses to apoptotic cells [118, 158]. It is well known that prompt apoptotic cell removal ensures anti-inflammatory and nonphlogistic responses by preventing cell lysis and harmful intracellular constituents release [122], inhibiting proinflammatory cytokine production and increasing anti-inflammatory one [52, 159]. This relates with the recruitment of monocytes. However, the find me signals are not specific for monocytes but they also induce the migration of neutrophils or others inflammatory cells.

Apoptotic cells also secrete keep-out signals, such as lactoferrin, to stop granulocytes recruitment [160].

Recognition and Tethering: Crosstalk Between Ligands, Bridging Molecules and Receptors

The apoptotic cell displays different molecules-ligands, called eat me signals, on plasma membrane for the recognition and subsequent engulfment by phagocytes. These signals include new molecules appearing on the surface, such as phosphatidylserine (PtdSer) or annexin I, modified existing molecules, such as changes to the surface molecules ICAM-3 and CD31, and changes in the charge of the cell surface [143, 161-163]. In parallel, a variety of macrophage receptors are involved in recognition and engulfment of apoptotic cells, including the integrins αvβ3 [164], αvβ5 [165], CD36 [166], a phosphatidylserine (PS) receptor [167], various lectins [127, 168], a scavenger receptor [169], CD14 [170], an ATP binding cassette transporter [171], LOX-1 [172] and CD68 [173]. "Eat me" signals and macrophage receptors can directly or indirectly interact *via* membrane-bound co-factors or soluble bridging molecules, such as thrombospondin (TSP1) [131], the Growth Arrest Specific gene product GAS-6 [174], Milk-Fat Globule Epidermal growth factor 8 (MFG-E8) [175], protein S [176] and β2 glycoprotein 1 (βGP1).

Among the surface changes occurring during apoptosis, PtdSer exposure is universally accepted as distinctive signal of an apoptotic cell [177]. The loss of phospholipid asymmetry and the traslocation of PtdSer from the inner to the outer leaflet of the plasma membrane have received the most attention and they are the well known and characterized modifications of cell surface in very early apoptotic process, independently both of cell types and death induction modality [178-180]. Normally, low levels flip-flop, inward and outward movement respectively, assure the asymmetric distribution of different lipids in the bilayer of the eukaryotic plasma membrane [181]. During apoptosis, the phospholipid flip-flop, also called scrambling, increases in coordination with the permanent inactivation of the aminophospholipid translocase, leading to persistent PtdSer exposure [180, 182-186]. Both the mechanisms and the nature of the molecules, proteins or lipids, mediating the processes of flip-flop and translocase inactivation are still unknown [183, 184, 187,188]. They are energy-, caspases activation- and oxidants production- dependent. In particular, the aminophospholipid translocase activity requires ATP but not calcium, conversely to phospholipid flip-flop [182-184]. Once externalized on cell surface, PtdSer can be recognized by different specific phagocyte receptors, various integrin and scavenger receptor families members [189]. The phagocyte PtdSer receptors can both directly and indirectly bind and recognize PtdSer exposed on apoptotic cell plasma membrane. The direct recognition occurs through members of the T cell Immunoglobulin and Mucin (TIM) family, such as TIM-1, -3 and -4 [190-196], Brain Angiogenesis Inhibitor 1 (BAI1) [197] and the atypical Epidermal Growth Factor (EGF)-motif containing membrane protein Stabilin-2 [198]. Conversely, the indirect binding to PtdSer includes MFG-E8, Gas6 and Protein S, and thrombospondin. In particular, MFG-E8, Gas6 and Protein S bind molecule PtdSer on apoptotic cells and integrin $\alpha_v\beta_5$ [175, 199] and Tyro-3-Axl-Mer (TAM) family receptors [200-202] on phagocytes respectively.

Besides PtdSer, a key recognition molecule on apoptotic cells, there are a lot of different molecules, e.g. aminosugars [122] or mannose [203, 204], intercellular adhesion molecule-3

[163, 205], lysophospholipids such as lyso-PC [141, 206, 207], or alterations in surface charge [122], related to changes in glycosyl groups, identified on apoptotic cells [208]. In addition, late apoptotic and necrotic cells expose molecules generally considered intracellular, such as annexin I, colocalising with PtdSer [209], ER protein calreticulin (CRT) [210] and DNA [211]. In particular, stressed cells increase the CRT surface expression also in association with new CRT synthesis [212, 213]. Deficiency in CRT exposure induces inefficient removal by phagocytes recognizing CRT by low-density lipoprotein receptor-related protein (LRP/CD91) [144]. Moreover, PtdSer and CRT colocalize on the apoptotic cell surface distributed in patches probably to optimize and drive ingestion by phagocytes [132].

Phagocyte Signaling and Engulfment

The internalization process profoundly depends on the involved phagocytic receptors. Particularly, after recognition, the microfilaments lead the engulfment while microtubules successively promote phagosome trafficking. In fact, soon after receptor-mediated recognition, actin polymerisation is observed to the site of ingestion driving pseudopods extension through cytoskeleton rearrangement. After internalisation, actin depolymerises and the phagosome matures by a series of fusion and fission events with endocytic components, also relies on the nature of the dying cell. The microtubules primarily drive the phagosome trafficking. The cytoskeletal reorganization is regulated by *CrkII*, *Dock 180*, *Rac* and *ELMO* (homologues to *ced-2*, *ced-5*, *ced-10* and *ced-12* respectively) [214-218]. Increasing evidences suggest that apoptotic and necrotic cells are engulfed by different modalities. Particularly, the apoptotic cells phagocytosis occurs in a "zipper"-like mechanism: the advance of the pseudopodia requires a sequential ligands-receptors interaction leading to a close-fitting phagosome without solute taken up during internalization. Conversely, necrotic cells engulfment, in a "trigger"-like process, only requires the initial interaction between ligands and receptors, able to induce cell surface ruffling ending in the formation of spacious macropinosomes containing both dead cell and external solute [219].

Processing of the Ingested Cargo and Its Degradation

Phagosome maturation depends on the dying cell nature, engulfment mechanism and/or recognition receptors involved. Mature phagosome membrane composition and contents are modified after interaction with endocytic compartment, as suggested by the distribution of acid hydrolases in the cell. Early endosomes contain cathepsin H, conversely, late endosomes contain cathepsin S [220].

The Toll-Like Receptor 4 (TLR-4) is very important in processing mechanism and negatively regulates engulfed cells degradation in macrophages [221]. Phagosome maturation of apoptotic cells is a slow constitutive process not influenced by signals received through TLRs [222]. Independently of the phagocyte species or the ingested cells, phagosomes containing apoptotic cells mature more rapidly than those containing opsonized cells in a Rho-mediated manner. The downstream targets of Rho kinase are Ezrin-Radixin-Moesin (ERM) proteins [223].

Release of Cytokines and Immunological Consequences

After internalization of dying cells, the phagocytes release molecules with functional consequences for immunity. Particularly, after apoptotic cell internalization, the macrophages secrete anti-inflammatory cytokines IL-10, TGF-β, Platelet Activating Factor (PAF) and ProstaGlandin E_2 (PGE_2) inducing pro-inflammatory responses suppression by direct autocrine and paracrine effects on pro-inflammatory cytokines TNF-α, GM-CSF, IL-12, IL-1β and IL-18 production [224-226]. It has been shown that TGF-β suppresses pro-inflammatory cytokines secretion by inhibiting p38 MAPK phosphorylation and $NF\text{-}_kB$ activation [227]. The anti-inflammatory effect of apoptotic cells also occurs by direct binding to macrophages, independently of phagocytosis or the involvement of soluble factors [228]. However, several studies suggest pro-inflammatory [229, 230] and/or immunologically silent [231] consequences of apoptotic cells phagocytosis, probably due to the macrophages (activation or differentiation state), target cells (source and activation state), type of cell death *stimuli* and presence of TLR ligands [232]. In this context, very crucial is the role of a class of molecules, named alarmins and also known as Damage-Associated Molecular Patterns (DAMPs), critical regulators of immunogenic cell death (see below).

In spite of apoptotic cell removal, necrotic cells recognition and internalization trigger pro-inflammatory macrophage responses [233, 234] by release of TNF-α, GM-CSF, IL-12, IL-1β and IL-18 pro-inflammatory cytokines. However, necrotic cells also trigger an inflammatory response by release of factors, such as High Mobility Group Box 1 protein (HMGB-1) [235], heat shock proteins [236], acid uric [237], genomic DNA [238], ATP and nucleosides [239, 240] leading to maturation and activation of macrophages and DCs.

The studies regarding macrophages responses after autophagic cells engulfment are very few. Petrovsky and coworkers [241] demonstrated that both professional, blood isolated human monocytes, and non-professional, MCF-7 cells, phagocytes engulf autophagic dead cells in a PS-dependent manner inducing the pro-inflammatory cytokine genes IL-6, TNF-α, IL-12B and IL-23B. The possible pro-inflammatory activity of autophagic cells could also be due to the exposure of pro-inflammatory signals on their surface [242], such as calreticulin [243], or to the induction of adenosine receptors expression on the macrophages, particularly ADORA2A, a potent regulator of macrophages functions [244].

Immunogenic Impact of PDT-Induced Cell Deaths

In classical terms, it has long been hypothesized that apoptotic cell death is poorly immunogenic (or even tolerogenic), whereas necrotic cell death is truly immunogenic. Nonetheless, it seems that this theoretical assumption is an oversimplification and does not withstand experimental verification, at least in models of tumour vaccination. Indeed, tumour vaccination studies in mice show that some apoptosis-inducing treatments cause immune-dependent tumour regression whereas others do not, pointing to a hitherto unsuspected heterogeneity in the biochemical pathways that in turn influences the immunogenicity of apoptotic cell death [245]. Indeed recent studies reveal that apoptotic tumour cells induced by anthracyclines [246] or ionizing irradiation [247] are able to induce a potent immune response *in vivo*, opening new questions on the pretended non-immunogenicity of apoptotic cell death.

Unlike apoptosis, necrosis has been and continues to be considered immunologically harmful because of the sudden release of pro-inflammatory mediators, such as interleukin-8 (IL-8), tumour necrosis factor-α (TNF-α) and high-mobility box-1 (HMGB1) protein [248].

On the other hand, the immunogenic impact of autophagic cell death is still a matter of debate [249]. Nevertheless, even autophagic cell death has been shown to be capable of stimulating some immunological flutters. Indeed, autophagy may influence the surface proteome of dying cells and stimulate the secretion of the immune modulator HMGB1, suggesting that it may be feasible to manipulate the immunogenicity of dying cells by increasing or decreasing autophagy [250].

The immunogenicity of the cells dying *via* apoptosis, necrosis or autophagy is mediated by a series of subtle changes in the composition of the cell surface and the secretion of soluble molecules allowing the immune effectors, primarily DCs, to 'sense' immunogenicity [251].

Intracellular molecules, categorized as DAMPs, also known as alarmins, normally hidden within live cells, are released from or exposed at the surface of dying cells determining the engulfment of apoptotic bodies, DC activation and maturation, antigen processing and T cell activation.

DAMPs exposed on plasma membrane (e.g. calreticulin, HSP70 and HSP90), or extracellularly secreted (e.g. HMGB1, uric acid, IL-1α and other pro-inflammatory cytokines) exert an immunostimolatory/immunomodulatory effect by interacting with pattern-recognition receptors (PRRs), including RIG-I-like receptors (RLRs), the NOD-like receptors (NLRs) and Toll-like receptors (TLRs), expressed on the innate immune cells [252]. The actual diversity of DAMPs, also including end-stage degradation products (e.g. ATP, DNA and RNA) and extracellular matrix compounds (e.g. hyaluronan, heparan sulphate and degraded matrix constituents), may depend on different factors, such as cell death modality, cell-type and tissue injury [253].

In terms of DAMPs, necrosis presents the most multi-faced immune-stimulating profile followed by apoptosis; conversely, autophagic cell death is characterized by a very actual diversity of alarmins. Particularly, the immunogenicity of apoptosis, whose immune-responsive profile has been hidden so far, critically relies on the early cell surface exposure of the Ca^{2+}-binding chaperone calreticulin (CRT), a protein normally found in the lumen of the ER. CRT exposure is potentially triggered by pharmacological cell death inducers (such as oxaliplatin or the anthracyclines, doxorubicine and mitoxantrone) able to trigger ROS production and ER stress response. This response requires PERK-mediated eIF2α phosphorylation and it is favored by ER-Ca^{2+} leakage [247, 254].

The appealing idea of immunogenic cancer cell death demands screening and selection of newer chemotherapeutic agents/modalities capable of sustaining a particular spectrum of DAMPs. PDT is a recent therapeutic modality endowed with a strictly association with certain DAMPs also on/by the immune system cells [255]. Particularly, PDT presents a remarkable beneficial immunomodulatory potential in terms of cancer disease management, since it is predominantly able in inducing expression, exposure/release of Heat Shock Proteins (HSPs) [5].

HSPs are a class of highly conserved proteins functioning as intracellular chaperones involved in the structural folding of both newly synthesized and stress damaged proteins. Extracellular and membrane bound HSPs, especially HSP70, are involved in binding tumour

antigens and presenting them to the innate immune cells, components of the immune system lacking of immunologic memory [252]. In particular, Korbelik and coworkes [256] reported cell surface expression and release of HSP70 in mouse SCCVII cancer cells photosensitized with Photofrin. Likewise, in HeLa and ASTC-a-1 tumour cells, Photofrin regimen induces HSP70 translocation to the outer leaflet of plasma membrane depending on PDT doses. Such translocation tightly correlates with the changes of mitochondrial transmembrane potential [257]. Extracellularly released HSPs also mediate an immunostimulatory effect [258]. Indeed, in the extracellular space, HSPs such as HSP70 and HSP90 might function as carriers of tumour-associated peptides or antigens interacting with APCs. Once processed and complexed to Major Histocompatibility Complex (MHC) molecules, HSP-peptide complexes could activate $CD8^+$ cytotoxic T lymphocytes to induce adaptive immune response [259]. Therefore, HSPs yield a link between innate and adaptive immunity.

The peculiar process mediating the PDT-induced adaptive immunity might be the massive phagocytosis of photosensitized dead cancer cells by innate immune cells providing for an efficient tumour-associated antigens presentation to adaptive immune cells [6]. The innate immune system, also known as non-specific immune system, comprises a series of immune cells involved in the first line of defence against infection [260]. It has been shown that PDT stimulates the recruitment of these cells into the treated area eliciting a local inflammatory response, sometimes accompanied by systemic neutrophilia, to cancer [261] (Figure 2).

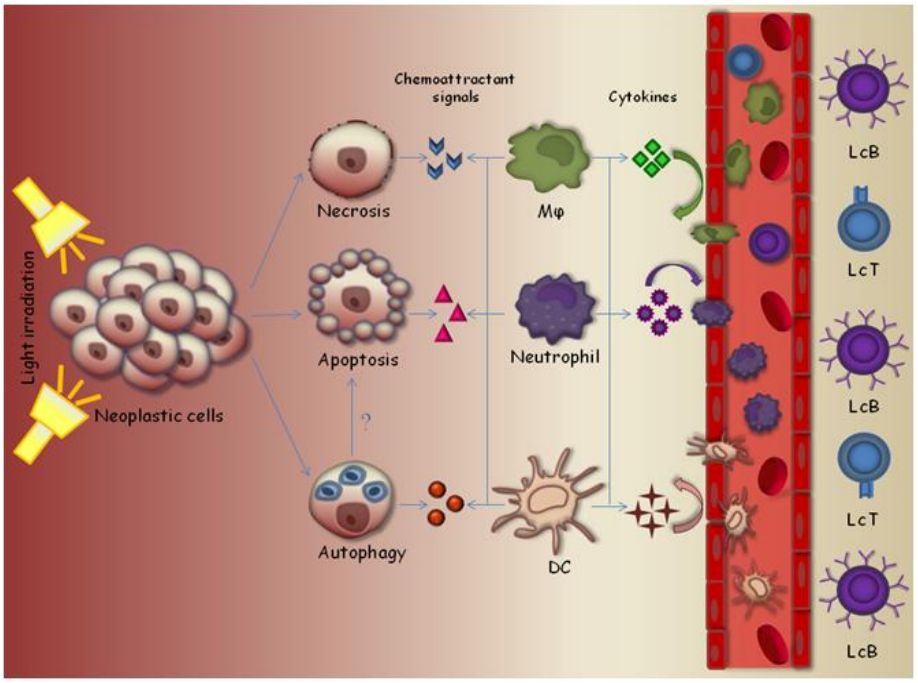

Figure 2. Photodynamic therapy induces inflammatory response. The irradiation of a PhotoSensitizer (PS)-loaded cancer cells commits to necrotic, apoptotic and autophagic cell death. Dying cells release chemoattractants signals recruiting phagocytes, including macrophages (Mφ), neutrophils and dendritic cells (DCs). These cells phagocytose photokilled cells triggering inflammation by cytokines release that in turn amplifies the further coming of immune cells. PDT also stimulate an adaptive immune response by activation of B (LcB) and T (LcT) cells recruited to the site of inflammation.

Photodynamic treatment is also capable of activating other immune phenomena, including acute-phase response, complement cascades and release of secondary inflammatory mediators, such as cytokines/chemokines. Therefore, upon irradiation, the photosensitized tumour microenvironment undergoes several changes, affecting mainly the activity of innate immune cells, that facilitate the development of anti-cancer adaptive response. Particularly, several preclinical and clinical studies highlighted that PDT, an oxygen-consuming treatment, activates the Hypoxia-Inducible Factor (HIF), the major oxygen homeostasis regulator activating genes essential to hypoxia cellular adaptation [262], in the tumour microenvironment [263].

Recent observations suggest that myeloid cells are evolutionarily adapted to function in low oxygen levels conditions [264]. In these cells, HIF regulates the secretion of several inflammatory mediators (e.g. TNF-α, IL-1, IL-12, nitric oxide) or coordinates the induction of Toll-like receptors TLR2 and TLR6 during hypoxia [265, 266]. On the other hand, HIF enhances the antigen presentation activity of DCs, by regulating the production of cytokines and co-stimulatory molecules [266, 267].

PDT immunomodulation entails the activation of macrophages, key players in the immune response. Macrophages are phagocytic cells derived from the differentiation of blood-borne monocytes in tissues that express a lot of cell surface receptors mediating their interactions both with natural and altered-self components of the host and a range of microorganisms [268]. Low sublethal PDT doses induce the macrophages activation [269], which produce the cytokine Tumour Necrosis Factor (TNF) mediating PDT cytotoxicity *in vivo*, either directly by TNF-mediated tumour necrosis, or indirectly by vascular effects on tumour vessels [270]. Krosl et al. reported an increased cytotoxic activity of tumour-associated macrophages against lethally irradiated murine squamous carcinoma cells (SCCVII) genetically engineered to produce murine Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), which amplifies the PDT-induced antitumour immune reaction [271].

Tumour-infiltrating neutrophils, other mediators of the innate immunity, heavily contribute to the onset of antitumour immunity following PDT. Indeed, they are retained among the first innate immune cells entering PDT-treated tumours. Krosl et al. [272] reported that Photofrin-based PDT induces a rapid increase in the content of neutrophils (200-fold within 5 minutes after the start of light treatment) in the squamous cell carcinoma SCCVII. Neutrophils adhere to the microvascular wall within 5 minutes after irradiation of Male Sprague-Dawley rats treated with Photofrin [273]. In the illuminated tumour area, these immune cells produce leukotrienes, prostaglandins, pro-inflammatory cytokines participating in the recruitment and activation of other immune cells types, and lysosomal enzymes, including MyeloPerOxidase (MPO), which destroy the residual cancer cells and the vasculature.

The primary importance of neutrophils invasion into PDT-treated tumours is highlighted by the study performed by Korbelik and Cecic [274] reporting that the selective immunodepletion of neutrophils population in EMT6 sarcoma-bearing mice resulted in a reduced curative outcome of Photofrin-based PDT. In agreement with this finding, another evidence underlining the indispensable role of neutrophils for successful PDT *in vivo* was published by de Vree and colleagues [275]. This report demonstrates that PDT does not retard tumour growth after administration of the anti-neutrophil serum to Photofrin II treated rhabdomyosarcoma-bearing rats.

The prominence of PDT-induced neutrophilia in generating a systemic anti-tumour immunity critically relies on the capability of neutrophils to stimulate the maturation of DCs, the most potent professional APCs acting as messengers between the innate and adaptive immunity [276]. DCs are immunological peripheral sentinels specialized in the uptake, processing and presentation of antigenic material and the detection of a broad *spectrum* of 'danger' signals.

DCs exist in both ‘immature’ and ‘mature’ states [277]. In absence of inflammation, DCs remain in the immature state; pro-inflammatory signals deriving either from microbes or dying cells induce a rapid change in their function, also known as ‘activation’. Activation entails several changes in DCs ending in their migration into local lymphoid tissue where they communicate antigenic information to lymphocytes [278]. Mature DCs express peptide-MHC complexes and co-stimulatory molecules on the cell surface enabling the initiation of the adaptive immune response. PDT is capable of inducing DCs activation. Indeed, PDT-generated tumour (murine EMT6/P815) cell lysates are able to provoke phenotypic maturation of DCs, activating them to express IL-12, MHC class II and co-stimulatory molecules (CD86). Due to their immunogenicity, these lysates may have clinical implication as effective antitumour vaccines [36].

Natural Killer cells (NKs) also participate in PDT-induced immune control of cancer since it has been shown that, although these cells are unable to directly kill EMT6 mammary fibrosarcoma tumour cells, NKs depletion with anti-asialo-GM1 antibody significantly reduced PDT efficacy in benzophenothiazine treated mice [279].

PDT induces local inflammation also leading to adaptive immunity development. Antigen-specific B and T cells provide the adaptive immunity. Particularly, B cells produce immunoglobulins helped by T cells to make antibody. PDT-treated tumour cells release antigens successively phagocytosed by DCs and presented to T cells in regional lymphonodes. Activated T cells return to the circulation and track down and destroy tumours [280].

Canti and coworkers [281] report the first evidence on the involvement of cytotoxic T cells in PDT anti-tumour effects. Immunosuppressed and normal mice bearing MS-2 fibrosarcoma were subjected to surgery or to aluminum disulfonated phthalocyanine (AIS2Pc)-PDT. Only surviving animals cured by PDT show resistance to MS-2 rechallenge.

CD8+ CTLs adoptive transferred from BALB/c mice cured of EMT6 tumour by Photofrin-PDT, in SCID mice EMT6 affected, provide curative effects. CD4+ helper T cells only play a supportive role [37, 38, 282].

The immune system can tackle tumours by recognition of Tumour Associated antigens (TAs) presented by MHC class I molecules on tumour cell surface inducing CTLs to destruct cancer cells [283]. It has been demonstrated that PDT induces a highly potent antigen-specific systemic immune response able to cause regression of micrometastasis not irradiated by light during photodynamic treatment [284, 285].

The role of TAs in PDT immunity in clinical field has been studied by Kabingu et al. [286]. The hedgehog-interacting protein 1 (Hip 1), a TA basal cell carcinoma lesions antigen, significantly increases when the lesions were treated with (ALA)-PDT or Porfimer sodium-PDT respect to surgically removed lesions.

Among CD4+ T cells, a special population, called Regulatory T cells (Tregs), suppresses immune responses by multiple pathways [287] regulated by immunosuppressive cytokine TGF-β [288], that in turn induces further proliferation of Tregs [289].

In addition, Tregs control T-cell activation by blocking DC binding to self-reactive T cells [290, 291]. Tregs may play an important and negative role in PDT anti-tumour immunity. In fact, low dose of cyclophosphamide (CY) and low dose CY combined with BPD-PDT deplete Tregs and trigger resistance to tumour rechallenge. Moreover, this combined treatment leads to a long-lasting immune memory [280].

Conclusion

In therapeutic terms, PDT holds great promise as cancer treatment modality since it is capable of both killing malignant cells by all three cell death types (apoptosis, autophagy and necrosis) and concomitantly stimulating stronger host anti-tumour response in one therapeutic paradigm. These properties rely on PDT ability to induce cell death associated with particular DAMPs, secreted or exposed on plasma membrane, mediating the immunogenicity of dying tumour cells. PDT-associated DAMPs can be exploited in the design of anti-tumour vaccines based on the administration of photosensitized tumour cell lysates generated by ex vivo PDT. The ability of PDT to elicit immunomodulatory effects also entails an efficient clearance of dead cells by professional or occasional phagocytes.

At present, very few studies have been performed in order to understand the phagocytosis mechanism involved in removal of photosensitized tumour cells, although it has been demonstrated that PDT stimulates the rapid recruitment and activation of a lot of immune cells, e.g. macrophages, DCs, NKs and neutrophils, by inducing an acute inflammation in the tumour microenvironment.

Further investigations both in vitro and in vivo are required to clarify all potential variables that could mediate the development of an anti-tumour immunity induced by this cancer strategy.

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Chapter 4

Photodynamic Treatment of Actinic Keratosis Using Ameluz®: Recapitulation of Clinical Phase III Studies in the Light of Novel Preclinical Research

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Abstract

In photodynamic therapy (PDT) of neoplastic skin lesions, both ALA and MAL penetrate skin cells and serve as precursors in the synthesis of the photosensitizer, protoporphyrin IX (PpIX), in tumor cells. There has been a long debate on the differences between ALA and MAL regarding their clinical efficacy, adverse effects and molecular mechanisms underlying their cellular uptake and metabolism. Application site pain is one of the most disturbing side effects of PDT. Conflicting results were published regarding the severity of pain caused by ALA- and MAL-PDT. Also, some authors argued that MAL may display higher selectivity for tumor cells than ALA.

Here, we summarize a very recent multinational, multi-centre, prospective, placebo-controlled phase III study comparing an ALA (BF-200 ALA, marketed as Ameluz®) and a MAL preparation (Metvix®). We assess the clinical results in view of new preclinical research that explores the molecular mechanisms of ALA and MAL uptake and the action of PpIX in normal keratinocyte and keratinocyte tumor cell lines and nociceptive

neurons. Particularly relevant are new results on the interaction of keratinocytes and nerve cells during PDT.

The phase III study comparing Ameluz® and Metvix® demonstrated strongly superior efficacy of Ameluz® over Metvix®, in both the primary and secondary read-outs total patient and total lesion clearance. However, application site pain was not elevated along with the increased efficacy.

The better efficacy of Ameluz® may be based on the strongly improved skin penetration and PpIX formation that was demonstrated on normal pig skin. It may be argued that MAL forms less PpIX due to its supposedly higher tumor selectivity. However, the comparison of PpIX-formation in tumor and non-tumor keratinocyte cell lines loaded with ALA or MAL showed that both precursors produced up to 6-times higher PpIX levels in tumor cells than in non-tumor cells. In all cell types, PpIX synthesis was considerably faster with ALA than with MAL. Since both compounds are not sufficiently hydrophobic to diffuse through biological membranes, both must enter the cells via transporters, one of which is the GABA transporter (GAT)-3. The similar selectivity for tumor cells is reflected by the clinical results, where application site effects after PDT with Ameluz® are very similar to those after Metvix® PDT.

With respect to the burning pain experienced by many patients during and briefly after the PDT illumination, we recently demonstrated that ALA and MAL enter sensory neurons. A GAT-3 type transporter is involved in both ALA and MAL uptake into neurons. Further investigations of cellular events occurring during PDT and potentially causing sensory neuron activation revealed (i) a direct activation of neuronal voltage gated calcium and sodium channels by PDT-derived reactive oxygen, and (ii) an indirect activation of nociceptive neurons by acetylcholine secreted from keratinocyte tumor cells during PDT. Since only the ultimate nerve endings are exposed to ALA or MAL in the epidermis, this second mechanism seems most relevant for the generation of painful sensations during PDT. Since acetylcholine is increasingly produced in keratinocytes during their migration towards the upper layers of the epidermis, the enhanced penetration of Ameluz® may indeed lead to a better efficacy without increasing painful sensations, thus explaining the clinical results.

1. Three Challenges for Photodynamic Therapy in the Clinical Management of Actinic Keratosis

Photodynamic therapy (PDT) represents a clinically effective and minimally invasive treatment option for a variety of neoplastic and nonmalignant conditions (Agostinis *et al.*, 2011). As PDT relies on the application of a photosensitizing drug in combination with target area illumination with a light source, it has generated increasing interest in the field of dermatology (For reviews see Kennedy *et al.*, 1990; *Peng et al.*, 1997; Gold MH, 2007; McCormack MA, 2008). When treating cutaneous malignancies using PDT, drugs can mostly be applied topically. This allows rather specific spatial localization of the drug on the skin surface by local deposition of the photosensitizer drug. Also illumination can be performed unobstructed, as the skin surface is per se easily accessible for light. Still, photodynamic therapy in dermatology can face some hindrances. To overcome these or pass by them is the central challenge for PDT today.

A particularly well studied type of cutaneous lesion treated using PDT is actinic keratosis (AK) (Babilas *et al.*, 2010). These epidermal lesions, also classified as “carcinoma-in-situ”, are generally induced by extensive expose of skin to sunlight (Ko, CJ 2010). Clinically, these

lesions are described as discrete, premalignant, and intraepidermally localized and commonly occur on rather sun-exposed skin, as in the face or on the hands. While AKs are already endowed with true neoplastic characteristics (Ko, CJ 2010), they bear the risk to progress to invasive neoplastic lesions, namely squamous cell carcinomas (Braathen *et al.,* 2007). These tumour types are potentially metastatic and the initially localized lesion will spread into subepidermal layers and gather access to the bloodstream. In order to prevent the conversion from a rather benignant, locally treatable AK to a malignant systemic type of cancer, current international dermatological guidelines highly recommend the early attendance to AK. Photodynamic therapy has recently been ranked as a first-line treatment option for this particular purpose (Braathen *et al.,* 2007). While some drugs have already been approved for this, all have to face the above mentioned challenges. These challenges generally encompass: (a) selectivity, (b) drug or prodrug delivery and (c) an advantageous ratio of clinical efficacy versus side-effects.

PDT drugs for AK management often utilize protoporphyrin IX (PpIX) as the final photosensitizing compound, but rely on its synthesis from prodrugs inside AK cells (Ericson *et al.,* 2008). This approach bears a great advantage when addressing the selectivity problem. When using prodrugs of the endogenously occurring heme precursor PpIX, such as 5-aminolevulinic acid (ALA) and its methyl-ester (MAL), selectivity for neoplastic cells relies on two factors. One is the enhanced uptake of these prodrugs into neoplastic cells, the other is an enzymatic imbalance in tumor cells acting in favor of PpIX synthesis and hampering heme conversion (Van Hillegersberg *et al.,* 1992). Some studies have addressed this issue by comparing ALA und MAL efficiency in PpIX formation in cell cultures. Here, many of them could demonstrate superiority for ALA in PpIX production efficacy (e.g. Washbrook and Riley 1997, Uehlinger *et al.,* 2000; Gaullier *et al.,* 1997; Tunstall *et al.,* 2002; Rodriguez *et al.,* 2006 and Lee *et al.,* 2008). Additionally, cellular uptake routes seem to vary in different tumor cell-types and some authors have suggested divergent transport pathways for the structurally rather similar molecules ALA and MAL (Rud *et al.,* 2000; Rodriguez *et al.,* 2006). In order to shed light on this issue explicitly in the field of dermatology, our lab has conducted studies comparing healthy and malignant skin cells in their potential of PpIX synthesis from ALA and MAL. The results are presented below and may enhance the understanding of the different clinical characteristics experienced with ALA or MAL PDT.

A further challenge is the delivery of the rather hydrophilic molecules ALA and MAL through the *stratum corneum* in order to reach the neoplastic cells. Considerations in this field require different mechanistical approaches then those concerning selectivity and efficacy on a cellular level. As for MAL, it has been proposed that the addition of a single methyl-group to the acid residue of ALA might enhance lipophilicity sufficiently to allow passive membrane diffusion (Gederaas *et al.,* 2001). This may possibly mean a loss of selectivity towards neoplastic cells. Nevertheless, one advantage of MAL utilization is its increased stability in aqueous solution at physiological pH (Kaliszewski *et al.,* 2007). This feature seems to be gained at the expense of PpIX forming capacity.

Ideally, one would include the superior PpIX-producing characteristics of ALA into a drug delivery system circumventing instability problems and enhancing the ability to overcome the *stratum corneum*. A recent report introduced the ALA-containing nanoscale lipid vesicle formulation BF-200 ALA, now marketed as Ameluz®, as such a drug (Maisch *et al.,* 2010).

A combination of an active ingredient with a delivery system like this may at first sight seem a "perfect match" for photodynamic therapy. But it still has to master the final challenge: Ensuring high efficacies while not producing elevated side-effects in the clinic. One of the most common side effects reported by clinicians is pain at the application site during illumination. As illustrated below, pain depends on various parameters. One might generally assume a straight correlation between efficacy and pain, thus, recent clinical and preclinical studies hold proof that additional factors influence this equation and show how improved penetration characteristics keep pain at bay, while ALA as the PpIX precursor drives efficacy to novel heights.

2. Two Sides of the Same Medal: Combining Clinical Experience with Preclinical Understanding

With the previous points in mind, it is worthwhile pondering how a novel medication for PDT fulfilling those challenges could be conceived, created and clinically put to test. This achievement could only stem from a combination of a biological understanding of the addressed matter and its transition into the clinical setting with respect to the urges and needs of patients and health care professionals. Thus, the novel clinical data describing Ameluz® as a valuable drug for PDT in dermatological practice stand on solid scientific ground formed by intense preclinical and basic research.

3. Clinical Impressions: Efficacy and Safety

Two clinical phase III studies were conducted with Ameluz® (then under its development code name BF-200 ALA), in order to test it for clinical efficacy and safety. The studies comprised 122 (Szeimies *et al.*, 2010) and 571 (Dirschka *et al.*, 2012) patients. The second study is to our knowledge the biggest prospective, randomized, multicentric and placebo-controlled study on PDT for Actinic Keratosis to date.

Patients included in these two phase III trials suffered from 4 to 8 mild to moderate AK lesions on face or scalp. In these studies, treatment was performed by treating the prepared lesions with Ameluz® or placebo (Szeimies *et al.*, 2010) or Ameluz®, Metvix® (a commercially available MAL-cream, Galderma) or placebo (Dirschka *et al.*, 2012) for 3 h followed by an illumination using either broad-spectrum or LED light sources. With broad spectrum light sources the total light dose was 75 – 200 J/cm², while it was 37 J/cm² using narrow spectrum LED devices. Lesions were assessed 12 weeks after the first PDT and remaining lesions, if present, were retreated. Finally, 12 weeks after the final PDT the number of patients with all lesions cleared and the total number of cleared lesions were determined.

As the first clinical trial, a randomized, double-blind, prospective, placebo-controlled phase III trial with 122 patients compared Ameluz® to placebo (Szeimies et al., 2010). The light sources used were either narrow-spectrum or broad-spectrum lamps. The patient complete clearance rates were significantly higher for Ameluz® than for placebo (66.3% versus 12.5%, respectively), and the lesion complete clearance rates were 81.1% versus 20.9%, respectively. The use of narrow-spectrum lamps emitting light at 630 +/- 9 nm

resulted in higher efficacy than that of broad-spectrum lamps (96% versus 53%, respectively, for total patient clearance, and 99% versus 70%, respectively, for total lesion clearance). Thus, an almost complete clearance could be achieved when the combination of Ameluz® and narrow-band PDT lamps was applied. However, the use of narrow-band lamps also resulted in stronger side effects. While in an overall counting, 59.3% of the patients reported pain during PDT using Ameluz® during the first PDT session (Placebo: 6.7%), this percentage was reduced to 24% during the second PDT session. When using broad spectrum lamps, 37.5% of the patients reported pain during illumination in the first PDT session, this value sunk to 19% during the second session (with placebo values of 11.8 and 6.3% respectively). Using narrow spectrum lamps, now proven to be more efficient in therapeutic outcome, the percentage of patients reporting pain was highest, reaching 85.1% and 50% during the first or the second PDT respectively.

In a second pivotal phase III trial (Dirschka et al., 2011) Ameluz® was compared with Metvix® and placebo in an investigator-blind setting. 571 patients were treated with photodynamic therapy using Ameluz®, comparator Metvix®, or placebo at a ratio of 3:3:1. Again, narrow-band and broad-band light sources were used.

The primary medical endpoint was the complete recovery of all of a patient's lesions 12 weeks after the last PDT. On average with all lamps, Ameluz® (78.2%) was significantly more efficient than MAL (64.2%) and placebo (17.1%). In addition, the total lesion clearance rates were higher for Ameluz® (90.4%) than for Metvix® (83.2%) and placebo (37.1%). This second study confirmed that the use of narrow-spectrum lamps resulted in higher efficacy, accompanied by stronger side effects. Using this type of lamps, the total patient clearance with Ameluz® was 84.8 %, with Metvix® 67.5 % and with Placebo 12.8 %. The total lesion clearance rates were 93.6 % and 89.3 % for Ameluz® and Metvix® respectively. Despite the significantly increased efficacy of Ameluz®, the frequency of pain was not different between Ameluz® (69.4%) and Metvix® (72.8%).

Pain intensity measured on an 11-point numeric rating scale (VAS score) was not significantly different between Ameluz® and Metvix®. Ameluz® PDT (in the first session for an AK in the face or forehead) resulted in an overall VAS score of 4.1 ± 3.42, while a Metvix® PDT for the same indication caused a VAS score of 4.3 ± 3.43.

The clinical studies asses a very good therapeutic profile for the novel PDT drug Ameluz®. While it proved to be superior to its competitor Metvix® at the primary study endpoint, it nevertheless did not present an augmented pain profile.

One might generally speculate that clinical efficacy and side-effect frequency would be somewhat proportional, i.e. a more efficient therapeutic approach would cause more ROS in the target area, elicit more cell death and thus cause more side effects in line with these events. This correlation can clearly be seen when the clinical data for broad and narrow spectrum lamps are analyzed. However, Ameluz® features an increased efficacy towards Metvix® without the parallel increase in pain during illumination as most disturbing side effect. This suggests that other factors than just the enhanced PpIX formation may be causative of this complex phenomenon. The preclinical investigations presented in the following sections may aid to solve this puzzle.

4. Preclinical Explanations: Molecular and Cellular Mechanisms

Photodynamic therapy using ALA and MAL takes advantage of an evolutionary old and well conserved biochemical production chain – the heme pathway, giving rise to heme as the prosthetic group in hemoglobin and to cytochromes, present in the respiratory chains of all mitochondria. Therapeutic exploitation of this pathway usually starts by circumventing the rate limiting step of this process, ALA-synthase, the enzyme that creates ALA from glycin and succinyl-CoA. When adding extra ALA to a cell's metabolism, the end product feedback inhibition (heme to ALA-synthase) is passed by, and heme s consequently synthesized according to the potential of the subsequent enzymatic cascade (Peng *et al.,* 1997; McCormack MA, 2008). Thus, the production of PpIX - the actual photosensitizer - derived from ALA and other ALA-based PDT prodrugs, is possible in every somatic cell. This raises the questions how selectivity towards neoplastic cells is achieved in order to spare the surrounding healthy tissue?

Several studies have addressed the differences in prodrug uptake in healthy versus neoplastic cells. This *in vitro* approach allows focusing on cellular membrane uptake processes without having to consider additional parameters like tissue permeability. Thus, such studies greatly aid our understanding of the most basic processes. It has repeatedly been shown, that ALA and its methyl esters trigger higher PpIX formation in cells of neoplastic origin *in vitro* and *in vivo*. Two explanations for this phenomenon have been found. One is an increased activity of one enzyme in the heme synthesis pathway, namely porphobilinogen deaminase (PBG-D). This enzyme catalyses the deamination step from porphobilinogen to uroporphyrin III. The increased activity found in neoplastic cells will lead to an overall increase of PpIX formation (Kondo *et al.,* 1993; Leibovici *et al.,* 1988; Schoenfeld *et al.,* 1988). On the other hand, accumulation of PpIX is anything but the physiologically intended outcome of the heme pathway. Thus, after PpIX is built up, it is normally metabolized to heme through the addition of iron. Ferrochelatase is the responsible enzyme. This enzyme in particular was also shown to be functionally altered in neoplasms (el Sharabasy *et al.,* 1992; Peng *et al.,* 1997; van Hillegersberg *et al.,,* 1992). Its function is decreased, combining an already augmented anabolism of PpIX with decreased catabolic mechanisms. This finally renders the selectively increased build up in cancerous and pre-cancerous cells possible. An attempt to explain this is that cells with generally increased metabolism – such as neoplastic or tumor cells – may form increased amounts of PpIX due to a higher mitochondrial activity (Calvazara-Pinton *et al.,* 2007).

Still, this is just part of the truth. Before ALA or one of its derivatives may enter the pathways described above, they have to be taken up by the cells, and uptake routes seem to vary greatly between cells of different origin. Additionally, divergent pathways were described for ALA and methyl-ALA. While longer chain ALA-esters reach sufficient lipophilicity to overcome cellular membranes via diffusion, this is not true for ALA and MAL, as they are both markedly hydrophilic (Uehlinger *et al.,* 2000). The use of a more hydrophilic molecule poses the problem of getting it transported through membranes actively, but it holds a big advantage: Membrane transition has to be regarded as a selectivity gate with the potential of distinguishing neoplastic and healthy cells. Thus, prodrugs that do not unselectively pass any membrane in reach, may be advantageous since they gather selectivity

via transport processes. Various transporters have been described to carry ALA and MAL (Doering *et al.,* 1998; Rud *et al.,* 2000; Gederaas *et al.,* 2001; Rodriguez *et al.,* 2006; Frølund *et al.,* 2010). But as stated above, it is important to analyze uptake in cell types typical for the addressed organ, in this case epidermal keratinocytes, to fully understand the therapeutic potential (Casas *et al.,* 2002). Thus, it was needful to conduct *in vitro* studies comparing healthy and tumorous keratinocytes in terms of uptake and PpIX formation. The results described here were collected in a very practically orientated *in vitro* system for comparing ALA and MAL as prodrugs in epidermal neoplasms.

Side-by-side studies in this system, comparing ALA and MAL uptake into healthy (CCD 1106 KERTr cells) and neoplastic keratinocytes (A431 cells from a squamous cell carcinoma) could reveal, that - regardless of the prodrug used - PpIX production is more pronounced in neoplastic keratinocytes by a factor of at least 4. With ALA even a factor of 5 is observed. Such differences in uptake rates and amounts of PpIX formation, indicating superiority of ALA in these parameters, have also been described in various other cells lines (e.g. Washbrook and Riley 1997; Uehlinger *et al.,* 2000; Gaullier *et al.,* 1997; Tunstall *et al.,* 2002; Rodriguez *et al.,* 2006 and Lee *et al.,* 2008).

Our studies also revealed that the accumulation of PpIX in A431 cells peaks faster and at lower prodrug concentrations with ALA than with MAL. Still, when prolonging MAL incubation times or increasing prodrug concentration, total fluorescence maxima do not differ greatly anymore (Schulten *et al.,* 2012). Thus, any clinical study in patients evaluating ALA versus MAL medication efficacy should be carefully interpreted and checked for differences in incubation times and concentration of the applied drugs. Both, selectivity for tumour cells and amount of PpIX formation can be influenced by the time of incubation. Tumour selectivity is higher with shorter incubation times, at the expense of lower overall PpIX levels in the tumour cells. Additionally, 5-ALA shows higher selectivity at lower concentrations (Schulten *et al.,* 2012). This may explain better tumour selectivity described for MAL over ALA, where the incubation time and prodrug concentrations were kept constant (Fritsch *et al.,* 1998).

In order to identify the relevant pathways for ALA and MAL uptake, experiments were performed with inhibitors and competitors of the uptake. For ALA, the most significant uptake pathway is the GABA transporter 3 (GAT-3), as the highly selective GAT-3 blocker (S)-SNAP-5114 is capable of blocking most PpIX formation in ALA incubated cell types, both normal and neoplastic. A GABA-associated uptake route had already been published for human and murine adenocarcinoma cells (Rud et al., 2000; Rodriguez et al., 2006). Additional transporters involved in ALA uptake may be amino acid transporters. MAL is also taken up via these transporters but their relative importance seems to be different from ALA (Gederaas et al., 2001; Schulten *et al.,* 2012). While in many cell types the GABA 3 transporter seems less relevant for MAL uptake, this is different in neurons where both ALA and MAL are taken up through this transporter (Novak et al., 2011).

Penetration depth into the epidermis is a fundamental parameter for PDT efficacy. The delivery of the rather hydrophilic molecules ALA and MAL over the *stratum corneum* into the vital epidermal layers represents a challenge for dermatological PDT medications. Regarding Ameluz® this challenge has been met combining ALA with a nanoscale lipid vesicle formulation. This formulation not only chemically stabilizes ALA, a molecule normally rather unstable in aqueous solutions at neutral pH (Kaliszewski *et al.,* 2007), but also greatly improves ALA transport into deep epidermal regions. While the detailed

biochemical mode of action is in this case still subject to detailed investigations, the enhanced penetration efficacy has been shown in a preclinical study by Maisch and co-workers in 2010. In this study, the penetration depth and speed of BF-200 ALA and a commercially available MAL cream (Metvix®) were compared. This study used PpIX formation in slices of the *ex-vivo* porcine skin samples as readout. Fluorescence was measured at different time points (3, 5, 8 and 12 h) after drug application. The collected data showed that BF-200 ALA leads to an appearance of PpIX fluorescence in much deeper layers of the epidermis. After the longest evaluated time point (12 h) BF-200 ALA induced PpIX fluorescence throughout the epidermis down to the basal membrane. This could not be seen after any time point using the MAL-containing cream which caused more superficial PpIX synthesis at all time points evaluated. No evidence for PpIX formation in the dermis were detected with either the ALA or the MAL formulations (Maisch *et al.,* 2010).

These preclinical findings are in good agreement with the phenomena observed in the clinic, namely the data from Dirschka *et al.,* 2012, which reported higher efficacy for Ameluz® in the clearance of actinic keratosis than with the MAL cream Metvix®. An increased penetration depth will in all likelihood lead to a more profound photosensitization of the AK lesions and therefore result in an increased clearance. Neoplastic cells can only endure and form lesions if they are born in the lower epidermal layers where proliferation takes place. Thus, successful drug treatment has to reach the *stratum basale*.

Yet, the side effect profile of Ameluz® appeared unaltered towards the one of Metvix® (Dirschka *et al.,* 2012). Side effects of ALA or MAL PDT are transient and mostly restricted to the application site. The most disturbing side effect in the clinics is application site pain during the illumination. Various theories have emerged on the source and molecular nature of pain during dermatological PDT treatments. Still, most of these theories lacked experimental proof so far. Combining clinical findings with recent results from basic research now allows a better understanding of the origin of pain during PDT illumination.

5. The Source, the Depth and the Transmission: Novel Insights into Pain during PDT

Many authors have described pain during PDT as a common side effect of differing intensity (Wiegell *et al.,* 2003; Kasche *et al.,* 2006; Moloney and Collins 2007; Gholam *et al.,* 2011). Both clinical studies reviewed above (Szeimies *et al.,* 2010; Dirschka *et al.,* 2012) found that the PDT light source correlates not just with treatment efficacy but also with the severity of PDT pain. A direct link between these two parameters may be assumed, such that a more efficient treatment may give rise to more singlet oxygen in the epidermis, causing elevated decay of neoplastic cells and a higher intensity of PDT pain. While this observation is rather intuitive, as it describes the proportional relationship of more light energy with stronger effects – both desired and unwanted - the results of the clinical study comparing the efficacy of Ameluz® and Metvix® did not hint augmented pain during treatment with Ameluz® (Dirschka *et al.,* 2012). This apparent discrepancy can be explained by studying the mechanism of PDT pain, in particular since the observation is contrary to previous publications where a lower pain profile was described for MAL than for ALA PDT (Kasche *et al.,* 2006).

Any treatment related pain arising during PDT will manifest itself in the primary sensory neurons innervating the epidermis. Epidermal nerve endings stem form pseudounipolar neurons in the dorsal root or trigeminal ganglia that send processes into the periphery and propagate excitations to the dorsal horn in the spinal cord or the trigeminal nucleus caudalis (Lumpkin and Caterina, 2007). Some of these neurons, mainly those giving rise to Aδ and C fibers are specialized nociceptors, i.e. detectors for noxious stimuli, capable of perceiving various damage associated events (Julius D., 2001). During PDT these neurons must somehow be activated - a necessary prerequisite for acute pain.

From a theoretical point of view, two pathways are conceivable for neuronal excitation through PDT. Singlet oxygen is formed in the target cells during PDT. Thus, if neuronal processes in the epidermis could also be considered as cellular targets, one explanation could be a direct neuronal activation during PDT. This theory has been formulated previously by authors attempting to speculate on molecular explanations for their findings that ALA PDT appeared clinically more painful than MAL PDT. The assumption was often that ALA, but not MAL, would enter sensory nerve endings, induce local PpIX production and illumination would then be the reason for neuronal excitation. These assumptions were based on the finding that in an adenocarcinoma cell line ALA but not MAL was taken up via GABA transporters (Rud et al., 2000).

Although only sparse data was available on the expression of GABA transporters in primary sensory neurons, this particular theory reached some acceptance in the community. Very recent findings from our own laboratory now greatly question the coherency of this theory (Novak et al., 2011). Using a rat primary sensory neuron model *in vitro*, we showed that both, ALA and MAL are taken up into sensory neurons and are capable of forming PpIX there. More detailed observations on the responsible uptake pathway also showed that the GABA transporter 3 (GAT-3) presents the common uptake route for ALA and MAL in these cells. When discerning the various nociceptor cells types, our studies provided evidence that also C-fiber neurons, that reach deep into the epidermal layers *in vivo* (neurons positive for the markers CGRP and isolectin B4 (IB4); Stucky and Lewin 1999; Priestley *et al.*, 2002) are capable of PpIX production. While PpIX synthesis after ALA application was faster than after MAL application, the maximally observed synthesis amount was nearly the same (Novak et al., 2011). Thus, when comparing clinical data on pain during PDT, close attention has to be paid to the study parameters, since shorter incubation times might lead to reduced pain using MAL (Novak et al., 2011).

Following this direct path of pain formation during PDT, one might wonder how ROS production in nociceptive nerve endings might lead to neuronal excitation. To gather data on this issue, we have turned to calcium microflurimetry using Fura—2/AM in cultured rat sensory neurons loaded with PpIX by ALA incubation. While these neurons could still respond normally to physiological depolarization stimuli with a calcium influx, they also responded to 10 minute blue light (380 nm) illumination (*in vitro* PDT) with a massive rise in intracellular calcium. Control neurons that were not incubated with ALA showed no such reaction. The level of the calcium rise was within the range of calcium transients seen by depolarization.

To further analyze the mechanisms involved, we investigated the source of the calcium rise. By omitting extracellular calcium during *in vitro* PDT, we demonstrated that extracellular calcium, and not intracellular stores, was mainly responsible. By using various

channel blockers we furthermore confirmed that voltage gated calcium channels (VGCCs) were involved in this process.

Blocking L-type, T-type and P/Q-type Ca2+ channels reduces the calcium influx caused by *in vitro* PDT to 25% of the value without VGCC blocking. Further investigations shed light on the question whether VGCC opening is directly caused by PpIX derived ROS or whether a membrane depolarization might be the driving force. Omitting extracellular sodium, physiologically responsible for action potential generation via voltage gated sodium channels (VGSCs), led to a 50% reduction of neuronal calcium responses to *in vitro* PDT. This argues in favor of a depolarization associated process underlying the neuronal activation to PDT derived ROS (Novak *et al.,* unpublished data). Cytosolic calcium alterations had already earlier been found as a consequence of photodynamic action in a variety of other cell types (for a review see Almeida *et al.,* 2004). While some studies attributed this to a depletion of intracellular stores (Cui *et al.,* 1997; Ricchelli *et al.,* 1999; Granville *et al.,* 2001), others described calcium influx over the plasma membrane (Specht and Rodgers 1991; Joshi *et al.,* 1994; Gederaas *et al.,* 1996; Tajiri *et al.,* 1998). Although few researchers had so far focused on the molecular pathway of this phenomenon, there is some additional evidence that cell membrane calcium channels are a potential influx route (Joshi *et al.,* 1994; Hill and Schaefer 2009). Also, evidence exists that neuronal calcium channels undergo modifications when exposed to ROS (Todorovic *et al.,* 2001; Annunziato *et al.,* 2002). Neurons depend greatly on a tight regulation of cytosolic calcium.

Any disturbance leads to altered neuronal activity, transmitter release, energy balance, excitability and ultimately death (Bergmann and Keller 2003; Berridge MJ, 1998; Svichar *et al.,* 1998; Thayer *et al.,* 2002; Vanden Berghe *et al.,* 2002). Studies on invertebrate neurons (Uzdensky et al., 2001; Uzdenskii *et al.,* 2008) as well as an early study on PpIX neurotoxicity in sensory neurons of chicken (Riopelle and Kennedy 1982) support our data as well as a report on the neurotoxic effect of the photosensitizer mTHPC in rat sensory neurons (Wright *et al.,* 2009). Thus, these new experiments are in good agreement with other published observations and add some functional knowledge on ROS biology in the peripheral nervous system in general.

These results may aid in understanding direct mechanisms leading to nociceptor activation during PDT and rule out the assumption that ALA is more pain inducing due to exclusive uptake into sensory nerve endings.

However, only the very final tips of the nerve fibers innervate the vital layers of the epidermis and represent a very limited area for PpIX synthesis directly inside these cells. Therefore, potential interactions between neurons and keratinocytes should also be given attention.

The interaction of nerve endings and keratinocytes in nociception is an important new aspect in cutaneous sensory biology (Chateau and Misery 2004). At least two types of C-fiber nociceptors project processes into the epidermis (Zylka *et al.,* 2005; Lumpkin and Caterina 2007), which terminate directly adjacent to the keratinocytes' membranes. Keratinocytes themselves also express nociception-associated receptors such as the ATP-sensitive $P2X_3$ channel or the TRPV1 receptor (Denda *et al.,* 2006). This enables keratinocytes to sense pain-related stimuli and communicate them via calcium waves through gap junctions and paracrine signaling via transmitter release to other keratinocytes or to the nerve endings (Koizumi *et al.,* 2004; Denda and Denda 2007; Tsutsumi *et al.,* 2009). One important messenger in neuronal activation is ATP (Koizumi *et al.,* 2004; Denda and Denda 2007). ATP is secreted in response

to mechanical, thermal and chemiosmotic stress, while it can be detected by the sensory neuronal ATP receptors $P2X_3$ and $P2X_{2/3}$ (Burnstock, G. 2000). Another promising candidate for keratinocyte-neuron communication is acetylcholine. Keratinocytes produce and release this transmitter (Grando *et al.,* 1993), which can be triggered for example by UV-light exposure and is involved in sunburns (Kurzen et al. 2007). Furthermore nicotinic acetylcholine receptor subunits as well as muscarinic acetylcholine receptors have been detected in dorsal root ganglion neurons (Shelukhina *et al.,* 2009; Cao *et al.,* 2011). Subunits of the nicotinic receptor were also documented in isolectin B4 (IB4) and Mrgprd positive non-peptidergic nociceptors (Khan *et al.,* 2003), a type of C-fiber neuron that deeply innervates epidermal layers (Dussor *et al.,* 2008; Dussor *et al.,* 2009).

We have therefore tried to analyze the functional impact of molecules secreted from a squamous cell carcinoma cell line treated with *in vitro* ALA-PDT on cultured rat sensory neurons. It turned out that the supernatant of A431 cells elicited responses in cultured sensory neurons that were visualized by calcium imaging with Fura-2/AM. These responses were only present when the A431 cells were subjected to the combination of ALA incubation and illumination. Such calcium signals in peripheral neurons were also observed, when the supernatants of PDT-treated A431 cells were transferred to the neurons. These signals were completely inhibited by a mixture of antagonists for the nicotinic (mecamylamine) and the muscarinic (atropine) acetylcholine receptors. This result strongly supports the hypothesis that acetylcholine is secreted from neoplastic epidermal cells upon PDT, and that this transmitter excites primary sensory neurons.

In order to interprete this finding with the clinical observations that (a) Ameluz® (ALA) PDT is more effective than MAL PDT in clinical studies, (b) Ameluz® (ALA) and Metvix® (MAL) show nearly the same pain profile during treatment, (c) Ameluz® drives PpIX formation in by far deeper epidermal layers than Metvix®, one has to consider the expression and function of acetylcholine in the epidermis.

Acetylcholine is present in the skin, where it serves multiple physiological functions. It is involved in keratinocyte barrier formation, migration, proliferation and differentiation. It thereby greatly regulates skin homeostasis via autocrine and paracrine signaling pathways (for a review see Kurzen *et al.,* 2007). As described above, acetylcholine release from keratinocytes is also triggered by UV-light. Kurzen *et al.* summarized the role of acetylcoline as an important messenger in epidermal responses to UV light exposure: Suprabasal keratinocytes are exposed to UV light, release acetylcholine which in turn activates basal keratinocytes to release nitric oxide. Nitric oxide causes an increased blood flow in adjacent vessels, thereby causing erythema. Excess UV light exposure cause sustained acetylcholine release, which disturbs epidermal homeostasis and leads to a loss of cellular attachment, blistering and keratinocyte cell death (Kurzen *et al.,* 2007). Translated to the PDT scenario, the identical mechanism might elicit PDT side effects, but rather than UV irradiation it is red light in combination with a photosensitizer that is responsible for this. Local pain, erythema and blistering are the major PDT side effects.

To finally close the circle to the clinical observations, it is important to understand that acetylcholine is spatially unevenly distributed throughout the epidermis. The most apical vital layers (granular and spinous) contain higher amounts of acetylcholine than the basal layer. The latter is almost devoid of the messenger and instead expresses acetylcholine esterase, the principal catabolic enzyme for acetylcholine (Nguyen et al., 2001). These authors also showed that acetylcholine triggers the final secretion processes in keratinocytes prior to their

programmed cell death and transition to corneocytes of the stratum corneum. This acetylcholine gradient may now be the ultimate key to understand why a PDT drug inducing PpIX until the basement membrane of the epidermis, in spite of higher efficacy, is not more prone to PDT side effects – especially pain – than a drug that mostly photosensitizes the apical layers of the epidermis. PpIX related pain seems to a major part related to induction by acetylcholine which is mostly secreted from the more superficial epidermal layers. Deeper penetration of the photosensitizer may thus improve clinical efficacy without causing additional nociceptor excitation.

Conclusion

Clinical studies have identified a novel ALA-containing nanoscale vesicle formulation (Ameluz®) as a valuable and efficient medication for the photodynamic treatment of actinic keratosis. The combination of deep epidermal penetration and the highly effective protoporphyrin IX prodrug ALA leads to extremely good clearance rates of AK lesions, especially when LED-lamps are used. Side effects on the one hand increase along with higher efficacy but are not augmented compared to a MAL containing cream of lower efficacy.

While deep efficient penetration of the entire epidermis is a key to high efficacy rates, exacerbated pain was not clinically observed. In an adequate *in vitro* setup comparing normal and neoplastic skin cells, ALA showed similar or higher selectivity towards neoplastic cells than MAL.

Pain differences between ALA and MAL PDT cannot be attributed to differential uptake of these compounds into sensory nerve endings, as preclinical data clearly showed that both PpIX prodrugs are transported into primary sensory neurons via the same transporter protein. Further preclinical studies revealed that acetylcholine plays a pivotal role in pain formation during PDT. Acetylcholine is distributed in a gradient through the epidermis, such that the uppermost vital layers show the highest synthesis.

Hence, this may be the spatial zone responsible for nociceptive processes during PDT that involve keratinocyte-neuron communication. Increasing penetration depth therefore seems mostly beneficial in terms of therapeutic outcome while it does not necessarily worsen the side effect profile.

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Chapter 5

Cellular Aspects of Photodynamic Therapy with Hypericin

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Abstract

Hypericin (HY) is a one of the promising naturally occurring photosensitizers that is known to induce apoptosis with high efficiency in various cell line models and has found applications also in photodynamic diagnostics solely due to its high specificity for tumor cells and tissues. Although vascular effects induced by photodynamic therapy (PDT) with HY (HY-PDT) has proved the most effective in many *in vivo* models, the mutual combination of vascular and cellular destruction is even more powerful. Similarly, apoptosis is generally accepted as the primary mode of cell death induced by HY-PDT, but deregulations of signalling pathways shifting the target cells to other modes of cell death are frequent and therefore studied intensively. These results indicate that the effect of the PDT at the cellular level is not just mechanistic and that the attributes of the target cells are at least important, if not crucial, for the efficacy of PDT. This chapter discusses multiple signalling pathways and mechanisms that seem to be significantly involved in cellular response to photocytotoxic insult of HY-PDT as well as various strategies to overcome emergent antagonistic effects and increase the overall efficiency.

Introduction

Photodynamic therapy (PDT) represents a relatively new but rapidly-developing anticancer approach based on administration of a non- or weakly-toxic photosensitizer and its activation with light of appropriate wavelength. It is known as a highly efficient modality for treatment of various cancerous and non-cancerous diseases and it fulfils the elementary

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theoretical requirements for successful cancer therapy such as selective destruction of neoplastic tissue with minimal toxicity towards normal healthy tissue (Dougherty et al., 1998).

Several levels of therapeutic selectivity that apply to the mechanism of PDT make it highly flexible and modular. The ability to evoke cellular and/or vascular effects as well as the ability to stimulate immune response makes it a multi-target therapeutic modality. The efficacy of PDT is directly affected by all three elementary components – the properties of photosensitizer, the spectral characteristics of light and its output power, and finally the presence of molecular oxygen. However, there are a number of other variables that have a positive as well as a negative impact on the overall toxicity of PDT, and therefore they are being studied extensively. As the direct action of the PDT and the consequent phototoxicity is limited by the irradiation time, the ability of cells to deal with the generated level of reactive oxygen species (ROS) and corresponding intracellular or extracellular stress, and to survive the intervention, affects among other things the overall efficiency of the treatment. There are further anticipated mechanisms, such as the structural attributes of cytoskeleton (Di Venosa et al., 2012), and those that are recognized as most significant will be discussed in this chapter.

Thanks to its efficiency, PDT is also finding its potential for applications in the treatment of microbial (Kharkwal et al., 2011) or viral infections (Harris and Pierpoint, 2011; Jacobson et al., 2001; Lim et al., 2012). However, PDT may be employed to improve anti-tumor efficacy of the oncolytic vaccinia virus (Gil et al., 2011) as well. It is also applicable for the treatment of actinic keratoses, for superficial, nodular basal cell carcinoma, for Bowen's disease, as well as some types of viral skin infections by human papilloma virus (HPV) such as verrucae of feet and hands, Condylomata acuminata, periungual warts, epidermodysplasia verruciformis, but also viral skin lesions (non-HPV related) such as molluscum contagiosum or herpes simplex (Rossi et al., 2009). Applications in this field of lesions are safe and successful; in comparison with other techniques PDT has fewer side-effects and less recurrence, but the most important property is that it is not invasive; this means a reduced risk of infections and excellent cosmetic results (Rossi et al., 2009). For example PDT with methylene blue has proved to be effective against herpes labialis with no side effects (Marotti et al., 2009). Anti-fungal PDT has been successfully employed against Candida albicans and other Candida species, and also against dermatophytes (Lyon et al., 2011). PDT with "Photosens" in virus-associated pre-cancer and early-stage cancer of the cervix uteri has demonstrated anti-viral effects in approx. 90% of cases (Trushina et al., 2008).

Basic Mechanisms

Although photosensitizer toxicity depends upon multiple factors, nevertheless the light of the appropriate wavelength and presence of oxygen are essential for its photodynamic action. Absorption of a photon that transforms the photosensitizer from a ground singlet state into an excited one eventually leads to the release of accumulated energy. Relaxation of the molecule back to the ground state might be accomplished either by emitting fluorescence that can be used in photodynamic diagnosis (PDD) for diagnostic purposes (Berg et al., 2005) or by intersystem crossing to a relatively stable (in the range of μs – ms) excited triple state followed by generation of radicals (Takemura et al., 1989). Relaxation from the triplet state

can generate either free radicals or radical ions by hydrogen atom extraction or electron transfer to biological substrates (such as membrane lipids), solvent molecules or oxygen (Berg et al., 2005; Henderson and Dougherty, 1992). The radicals generated by the photosensitizer can interact with ground-state molecular oxygen to produce superoxide anion ($O_2^{\bullet-}$) radicals, hydrogen peroxides (H_2O_2) and hydroxyl radicals (•OH) (so-called "Type I reaction"). Direct transfer of energy from the triple state photosensitizer to the ground state molecular oxygen forms non-radical but highly-reactive singlet oxygen ($^1O_2$) (so called "Type II reaction") which is of higher significance for PDT action (Niedre et al., 2002). On the other hand, production of superoxide anions in the Type I reaction can form hydrogen peroxide, which is able to diffuse through membranes and thus might be toxic for neighboring cells. Addition of another electron can lead to generation of two hydroxyl radicals (•OH), the most dangerous member of the reactive oxygen species (ROS) family with the ability to attack and oxidize any compound of biological origin (Halliwell and Gutteridge, 1984; Plaetzer et al., 2005). Another mechanism published, however poorly discussed, is an oxygen-independent photochemical action (so called "Type III reaction") when the photosensitizer reacts with biological substrates in the absence of oxygen (Laustriat, 1986; Sibata et al., 2000). Apparently the Type III reaction is of remote significance, since for example no alterations of cell survival were detected after PDT with hypericin (HY-PDT) in hypoxic conditions (Thomas and Pardini, 1992). Both oxygen-dependent reactions occur simultaneously, but the ratio between them depends on the photosensitizer and available substrate molecules (Berg et al., 2005), and therefore the outcome logically depends also on the intracellular localization (Ahmad and Mukhtar, 2000) as this affects different cell organelles and induces cell death with variable efficacy.

Under *in vivo* conditions, however, there are many more factors that affect the overall outcome of PDT. The structure of the vascular endothelium is one of the factors held responsible for photosensitizer uptake rates. For example, the highest concentrations of Photofrin injected into mice (sacrificed 24 h later) were found in the liver, and the lowest in the brain when tested under regular physiological conditions (Bellnier et al., 1989). The liver is known to have a highly permeable blood supply with a fenestrated endothelium that contains pores that allow molecules to pass easily out of the vessels, whereas the brain has a relatively impermeable endothelium. The blood-brain barrier serves to exclude the photosensitizer from the parenchyma of the brain, and the fact that the blood-brain barrier is frequently breached by the growth of brain tumors is probably responsible for the very large tumor-to-normal brain ratios (over 100) reported for some photosensitizers (Boyle and Dolphin, 1996). In cancer therapy, tumor vasculature and parenchyma cells are both potential targets of PDT damage. But the preference of vascular *versus* cellular targeting is highly dependent upon the relative distribution of photosensitizers in each compartment, which is governed by the photosensitizer's pharmacokinetic properties and can be effectively manipulated by the photosensitizer drug administration and light illumination interval (drug-light interval) during PDT treatment, or by modification of the photosensitizer molecular structure (Chen et al., 2006). Besides this passive targeting mechanism (dependent on innate photosensitizer physico-chemical properties), active targeting of various tumor endothelial and cellular markers has been studied extensively (Reddy et al., 2006).

The anti-vascular effects of HY-PDT were nicely introduced in a study of its efficacy and tissue distribution (Chen et al., 2001), when higher efficacy correlated with plasma concentration rather than tumor drug level. Neither tumor hypoxia nor increasing tumor

oxygenation could significantly affect the effectiveness of various PDT protocols. These results suggested tumor vasculature damage as the potential primary mechanism of the HY-PDT effect (Chen et al., 2001). Although tumor therapy based on targeting the vasculature of the tumor is indeed promising, as demonstrated in the higher relative regression percentage of the treated tumor compared to the cellular-targeted PDT (Olivo and Chin, 2006), Cavarga and colleagues presented another approach based on fractionated dosing of hypericin. They proved that hypericin administrated 1 and 6 hours before irradiation can produce a better therapeutic response than either single administration. Their results suggest that cellular and vascular toxicity (and perhaps immune response) are all involved in the overall therapeutic outcome (Cavarga et al., 2005). Whilst the acute destruction of tumor vasculature directly affects the oxygen and nutrition supply, leading to starvation, it can also induce cellular resistance by multiple mechanisms triggered, for example, by hypoxia or malnutrition. Cellular destruction of the tumor cell, however, is the final task that must be accomplished to achieve a successful therapeutic outcome. For this reason, cellular aspects represented by the molecular and physiological phenotype of the target cell are the key to its eradication, although not exclusively.

From a number of examples a comparison of sensitivity to hematoporphyrin monomethyl ether (HMME) mediated PDT brings straighforward evidence (Li et al., 2010). In this case, C666-1 nasopharyngeal carcinoma positive for Epstein-Barr virus (EBV) demonstrated a higher resistance related to lower uptake of the photosensitizer in comparison to EBV^{neg} C666-1 cells (Li et al., 2010). This proves that even small changes may result in different sensitivity of tumor cells to PDT.

Among those cellular aspects that seem to be significantly involved in mechanisms of PDT, the intracellular level and localization of the photosensitizer, the ability to handle increased levels of ROS, the activity of the particular cell-death related signalling pathways and their consequent resistance, or the ability of the cell to proliferate, are the ones of greatest significance.

Hypericin in PDT

Hypericin (1, 3, 4, 6, 8, 13-hexahydroxy-10, 11-dimethylphenanthro[1, 10, 9, 8-*opqra*]perylene-7, 14-dione), a naturally-occurring photosensitizer, is a naphthodianthrone synthesized by plants of the *Hypericum* species (e.g. St. John's wort). Among others it possesses properties suitable for PDT (Agostinis et al., 2002) and PDD (Ali and Olivo, 2002; Head et al., 2006; Thong et al., 2009). Peculiar attributes of this photosensitizer are high efficiency in production of singlet oxygen (Redmond and Gamlin, 1999) and superoxide anions after irradiation with light wavelengths around 600 nm, and low or no toxicity in the dark (Fox et al., 2001; Jacobson et al., 2001). Its spectral responsivity enables *in vivo* application of HY-PDT in the treatment of tumors with depths in the range of 1 cm (Blank et al., 2002). Photoactivated hypericin is known to induce apoptosis with high efficiency in various cell line models as well as changes at the vascular level, or even to affect CD8+ T cell-mediated cytotoxicity (Lavie et al., 2000). At the cellular level, activated hypericin induces many events that are more or less specific, such as membrane lipid peroxidation, (Chaloupka et al., 1999a; Kello et al., 2010) increased activity of superoxide dismutase,

decreased glutathione concentration (Hadjur et al., 1996) or injury to mitochondria (Vantieghem et al., 2001).

The Greeks and Romans had already documented the medical use of St. John's Wort (*Hypericum perforatum* L.) in the treatment of nerve-related disorders. It is one of the most commonly prescribed antidepressants (Pal and Mitra, 2006). Although the main active components of St. John's wort are thought to be hypericin and hyperforin (Chatterjee et al., 1998), there are also other common plant constituents (e.g., flavonoids and flavonoid derivatives, xanthone derivatives, amentoflavone, biapigenin, volatile oil) that may have antidepressant effects. However, hypericin and pseudohypericin are the main constituents with photocytotoxic attributes; although hypericin is proving to be the more efficient and more easily applicable photosensitizer, as pseudohypericin interacts irreversibly with serum constituents (Vandenbogaerde et al., 1998). Hypericin is used in PDT and PDD as a highly purified molecule as well as in mixtures with other molecules as extracted from St. John's wort. A polar methanolic fraction proved to have significant photocytotoxicity, selective localization, and natural abundance, but above all is an easy and inexpensive preparation (Skalkos et al., 2006; Stavropoulos et al., 2006).

Hypericin fluorescence has found its applications in detection of tumors via PDD using fluorescence cystoscopy of bladder cancer (Jichlinski and Leisinger, 2005). Sim and colleagues proved that PDD using hypericin has higher sensitivity but equivalent specificity compared to white light cystoscopy and has a high potential to detect small tumors overlooked by standard cytoscopy (Sim et al., 2005). Similarly, hypericin is also applicable for clinical diagnosis of oral cancer (Thong et al., 2009). It is also well tolerated with minimal side-effects. Hypericin has also affirmed *in vitro* activity against several viruses, including bovine diarrhea virus, a pestivirus with structural similarities to hepatitis C virus (HCV). However, hypericin given orally in doses of 0.05 and 0.10 mg/kg/d caused considerable phototoxicity but had no detectable anti-HCV activity in patients with chronic HCV infection (Jacobson et al., 2001). Its ability to inhibit various enzymes seems to be relatively specific. Hypericin, whether light-activated or not, has been reported to inhibit an extensive spectrum of Ser/Thr protein kinases, protein tyrosine kinases or even HIV-1 reverse transcriptase (Schinazi et al., 1990), and it also seems to play a role in multidrug resistance.

The cytotoxic effects of hypericin are generally considered as oxygen- and light-dependent, and this fact is supported by a robust amount of experimental data. For example, light-dose fractionation for the purpose of spheroid reoxygenation did not produce any change compared to a single dose; though hyperoxygenation invoked massive destruction throughout the spheroid and induced apoptosis across the whole section (Huygens et al., 2005). One example from the opposite camp involves absolute elimination of hypericin photocytotoxicity in a hypoxic environment (Delaey et al., 2000), together with zero effect on mitochondrial function (Utsumi et al., 1995a). But the formation of hypericin's radicals, and subsequent hypericin-induced pH drop, has been suggested as potential mediators of its photocytotoxicity as well (Agostinis et al., 2002; Chaloupka et al., 1999b). Moreover, inhibition of some enzymes has proved to be light-independent (Johnson and Pardini, 1998), and the anti-metastatic and cytotoxic activity of hypericin in the dark has been demonstrated both *in vitro* (Blank et al., 2001; Blank et al., 2003) and *in vivo* (Blank et al., 2004). Since the light-independent action of hypericin generally requires markedly higher doses, and it is applied in almost every experimental work as control in the dark, it is therefore mostly negligible at lower concentrations.

Table 1. An example of light-independent actions of hypericin

Effect	Details	References
antidepressant	inhibition of glutamate release in cerebrocortical synaptosomes via MAPK	(Chang and Wang, 2010)
EGF binding	inhibition of epidermal growth factor binding	(Richter and Davies, 1995)
EGFR activity	inhibition of epidermal growth factor receptor tyrosine kinase activity	(de Witte et al., 1993)
HER2 activity	inhibition of human epidermal growth factor receptor 2 tyrosine kinase activity	(Hwang et al., 2001)
p44ERK1 p42ERK2	light-independent inhhibion in vascular endothelial cell models	(Lavie et al., 2005)
PKC inhibition	suggestion on binding of HY to regulatory domain of PKC	(Kocanova et al., 2006; Takahashi et al., 1989)
$CD8^+$ T lymphocytes	inhibition of CD8+ cytotoxic T-lymphocytes by hypericin in nanomolar concentrations	(Lavie et al., 2000)
inhibition of angiogenesis	hypericin in μM-range inhibits key steps of angiogenesis including endothelial cell proliferation, tubular formation on Matrigel, extracellular matrix degradation by urokinase, migration and invasion	(Martinez-Poveda et al., 2005)
HIF-1α	hypericin enhances HIF-1α degradation in hypoxia by lysosomal cathepsin B enzymes	(Barliya et al., 2011)
ABC-transporters	hypericin induces activity of MRP1 and BCRP	(Jendzelovsky et al., 2009)
HSP90 ubiquitinylation	loss of HSP90's chaperone function resulting in degradation of p53, Cdk4, Raf-1, Plk	(Blank et al., 2003)

Intracellular Localisation

The direct effect of PDT at molecular level is based mostly on the attributes of singlet oxygen. Since the half-life of singlet oxygen (<0.04 μs) and radius of its action (<0.02 μm) are short, the molecular targets affected by the photodynamic action of the photosensitizer must lie within a few nanometers from the molecule itself (Moan and Berg, 1991). The primary site of photodamage and localization of the drug therefore generally coincide, and so the most frequent targets of photosensitizers are mitochondria, lysosomes, plasma and intracellular membranes, Golgi apparatus and endoplasmic reticulum (ER). Since most dyes do not accumulate in cell nuclei, PDT generally has a much lower potential of causing DNA damage, mutations and carcinogenesis compared to that of X-radiation at equitoxic fluencies/doses (Oleinick et al., 2002).

Photosensitizers localized in the mitochondria and ER tend to promote apoptosis, while those targeting the plasma membrane or lysosomes can delay or even block apoptosis, thereby increasing the predisposition for necrosis (Kessel et al., 1997). On the other hand, rapid relocalization of certain phototsensitizers to other subcellular locations shortly after irradiation has also been reported (Berg et al., 1991; Kessel, 2002; Kessel et al., 2005; Marchal et al., 2007), suggesting that besides the primary site, photodamage can be rapidly propagated to other subcellular locations. Some attributes of the other products generated by

photodynamic action or photosensitizer relocalization during activation therefore play their part in the overall toxicity of PDT as well. Most of the papers discussing hypericin intracellular localization demonstrate its preferential accumulation in the perinucleolar cytoplasmic area, mainly on one side of the nucleus, a region rich in endoplasmic reticulum and Golgi, sometimes in the nuclear envelope but not in the plasma membrane. Even so, hypericin has also been found in the intercellular spaces between tightly-contacting cells in colonies (Ritz et al., 2008; Uzdensky et al., 2001).

At the beginning of this chapter it was suggested that cellular uptake of hypericin is determined by diffusion and solubility, neither of which requires an energy-dependent transport process or binding to specific receptors (Thomas and Pardini, 1992). But subsequently data confirming that sensitizer internalization may be a result of three mechanisms, namely partitioning (Mitragotri et al., 1999), pinocytosis or endocytosis were presented (Siboni et al., 2002). It has also been demonstrated that the involvement of a particular internalization mechanism affects the subcellular targeting of the photosensitizer as well.

In this study of Siboni and colleagues (Siboni et al., 2002), lipophilic sensitizers (e.g. hypericin or 1,3,4,6-tetrahydroxyhelianthrone (Hel)) were localized in the endoplasmic reticulum after protein-free internalization, whereas a hydrophilic molecule (namely hypericin tetrasulfonic acid (HypS4)) was localized in the cytoplasmic membrane and in lysosomes. An experiment performed under serum-enriched conditions localized all three photosensitizers to lysosomes as a target of endocytosis. A higher level of hypericin in an LDL-enriched medium supplemented with serum (when compared to a medium supplemented with serum only) supports the endocytotic mechanism. The different uptake parameters of hypericin to cells, with or without serum, effectively reflect the different mechanisms. The interrelationship between subcellular targeting and photodynamic treatment has been shown for the three sensitizers, and hypericin was found to be the most efficient sensitizer for PDT under the given illumination protocol. The degree of cell damage was found to be subject to the hypericin subcellular localization, because the subcellular site of singlet oxygen production proved to be critical for cell survival (Siboni et al., 2002). This conclusion emphasizes the importance of hypericin localization, which may be of equal importance as the overall concentration (Siboni et al., 2002).

Lipophilicity and/or interaction with serum proteins have been shown to significantly affect subcellular localization and HY-PDT efficiency in multiple studies. Comparison of hypericin and its derivatives with increased lipophilicity (hypericin-acid hexylamide, -acid octylamide and -acid dodecylamide) has revealed that hypericin binds mainly to LDL (low-density lipoproteins), whereas increased lipophilicity leads to preferential affinity toward HDL (high-density lipoproteins). Accumulation within cells was higher with hypericin bound to LDL, though intraspheroidal permeation was higher with lipophilic derivatives bound to HDL. Experiments in a medium free of any supplements showed a higher accumulation than with the medium supplemented with serum, and lower accumulation with increased lipophilicity (Crnolatac et al., 2007). The role of LDL and the LDL receptor pathway was further supported by Kascakova and colleagues, who demonstrated that hypericin uptake was higher in cells with elevated numbers of LDL receptors, and hypericin was found in co-localization with lysosomes (Kascakova et al., 2008).

With regard to the elementary principle of PDT it is generally accepted that concentration of the photosensitizer is one of the factors limiting PDT efficiency. Another factor that may affect the efficiency of PDT is the activity of various redox systems. Some of them, such as manganese-containing superoxide dismutase (Mn-SOD), are found to be largely diminished in many cancers (Oberley and Buettner, 1979), as the expression of Mn-SOD seems to be unprofitable to cancer cells due to suppression of HIF-1α activity and VEGF expression (Wang et al., 2005). On the other hand, significant up-regulation of metallothioneins (MTs), a group of cysteine-rich protein thiols with a strong affinity for heavy metals and hydroxyl radicals (Hayashi et al., 2006; Satoh et al., 1994), (especially MT-1E and MT-2A isoforms) was linked with prevalence of necrosis after HY-PDT (Du et al., 2006b). Although MT expression in certain tumor cells has been associated with resistance to anticancer drugs (Satoh et al., 1994), the PDT-mediated up-regulation of MTs in tumor cells did not affect their sensitivity to PDT-induced necrosis. It appears that in this case the oxidative stress induced by PDT overwhelmed the antioxidant defense mechanism such as the alteration of MT levels in tumor cells (Du et al., 2006b). In line with these conclusions, it has been suggested that not only hypericin uptake, but importantly also the cells' ability to deal with oxidative stress induced by HY-PDT, can be important decisive factors finally affecting the cell death response (Mikes et al., 2011).

There is also evidence demonstrating that various types of ROS may play a role in cell death signalling, too. A model of pyropheophorbide-a methylester (PPME) photosensitized cells suggested that singlet oxygen ($^1O_2$) produced at the ER/Golgi membranes mediates necrosis, whereas ROS other than $^1O_2$ (e.g. $O_2^{\bullet-}$, H_2O_2 or ●OH) produced by PPME at the primary site act as second messengers causing the mitochondria to release cytochrome c and initiate the intrinsic apoptotic pathway via caspase-3 activation (Matroule et al., 2001).

In line with this idea an experiment with Mitotracker Red-loaded HeLa cells demonstrated that active production of ROS (mainly $^1O_2$ and $O_2^{\bullet-}$) by the mitochondria can be induced by various oxidative insults and can lead to necrosis (Chernyak et al., 2006). Zorov and colleagues named this event earlier as ROS-induced ROS release (RIRR) and described it as a result of exposure to excessive oxidative stress leading to an increase in ROS (Zorov et al., 2000; Zorov et al., 2005; Zorov et al., 2006). When ROS numbers reach a threshold level, the opening of one of the requisite mitochondrial channels follows, together with the simultaneous collapse of the mitochondrial membrane potential and a transient increase in ROS generation by the electron transfer chain.

The generated ROS can be released into cytosol and trigger RIRR in the neighboring mitochondria. This mitochondrion-to-mitochondrion ROS-signalling represents a positive feedback mechanism for enhanced ROS production leading to potentially significant mitochondrial and cellular injury (Zorov et al., 2006). Nevertheless, a mitochondrial permeabilization transition pore opening was not involved in ROS production or cell death in either the PDT or the H_2O_2 model of Chernyak and colleagues (Chernyak et al., 2006). On the other hand, inhibition of flavin-containing enzymes (indicating Complex I involvement) stopped ROS accumulation and incubation with a mitochondria-targeted antioxidant (MitoQ) and even blocked necrotic cell death, whereas caspase inhibition did not. Conversely, Mitotracker Red-induced apoptosis was not affected by MitoQ, but it was counteracted by Bcl-2 or caspase inhibition (Chernyak et al., 2006).

It is evident that the type of ROS and the site of their production within the cell together represent the vital death-switch mechanism which certainly participates in regulation of the transition between apoptosis and necrosis. The possibility of involvement of Complex I, a major source of superoxide ($O_2^{\bullet -}$) within the mitochondria, is also considerable in necrotic signalling.

Cell Death Signalling

Since PDT is a versatile approach, there are a number of papers discussing the photocytotoxicity of different photosensitizers, their concentrations and incubation times tested *in vitro* with various cell lines in dependence on several variables. Matching these particular results is complicated, mainly due to different light sources and their attributes; however, the tendencies are very similar. For example, Kamuhabwa and colleagues demonstrated that the type of cell death (apoptosis or necrosis) in AY-27 cells induced by HY-PDT changed as a function of fluency rate, light dose and hypericin concentration, but depends largely on the hypericin concentration and the post-irradiation time (Kamuhabwa et al., 2001). It has been suggested also by others that the balance between apoptosis and necrosis appears to be dependent on the overall dose of PDT, but also on other features such as the cell type, its genetic and metabolic potential, the nature of the photosensitizer and its sub-cellular localization (Piette et al., 2003).

Molecular mechanisms of hypericin photocytotoxic action have been intensively studied for almost two decades. Nowadays it is apparent that hypericin is capable of stimulating pathways leading to cell demise but also to survival, and like many other photosensitizers, it can induce apoptosis as well as necrosis or autophagy. If a very simplified, purely mechanistic model of photodynamic action is governed by a quantity of oxygen and by total PDT dose achieved by combination of light dose and hypericin concentration, then cellular damage should be directly proportional to total dose if not limited by the amount of oxygen. However real output, considered as the quality and quantity of cell death, is significantly case sensitive. As for all photosensitizers generally, hypericin's photodynamic action also depends on many distinct factors and creates a multifarious system in which some of those factors can be controlled.

It has been reported by many authors that the shift from apoptotic to necrotic cell death can be effectively produced by increasing the concentration of hypericin and/or the light dose applied to sensitize the cells (Kamuhabwa et al., 2001; Vantieghem et al., 1998; Vantieghem et al., 2001). It seems that in those cases cellular damage was in correlation with ROS level (Agostinis et al., 2002) and necrosis occurred at higher, toxic doses. On the other hand, low doses may have opposite effects resulting in photo-tolerance to subsequent high-dose PDT (Kulikova et al., 2011; Sackova et al., 2005).

The mode of cell death is also significantly governed by hypericin uptake and intracellular localization as well. For example, photoactivated photosensitizers with prevalently mitochondrial localization (e.g. porphyrogenic sensitizers and phthalocyanine-related compounds) rapidly mediate $\Delta\Psi m$ dissipation accompanied by cytochrome c release and a drop in intracellular ATP levels (Almeida et al., 2004; Oleinick et al., 2002). But the mitochondria are also critical executors of lethal pathways emanating from photodamage to

other subcellular sites or organelles, although in this case the release of apoptogenic proteins from the mitochondria may be delayed (Buytaert et al., 2007). Since hypericin is mostly reported to be localized in the endoplasmatic reticulum and/or Golgi apparatus as well as in lysosomes, it should induce apoptosis with lower efficiency (Marchal et al., 2005). In spite of this, rapid loss of ΔΨm, subsequent cytochrome c release, caspase-3 activation and apoptosis occur as a result of the photodynamic action of activated hypericin. Since the photocytotoxic action of hypericin represents a massive impact on different cellular targets, cytochrome c release as well as caspase-3 activation and apoptosis can be suppressed in cells over-expressing Bcl-2, but not the ΔΨm loss (Hadjur et al., 1996; Chaloupka et al., 1999b; Vantieghem et al., 2001). In accordance with the above-mentioned experimental data, double deficiency in *bax* and *bak* proved the essential role of Bak and Bax in ΔΨm dissipation and the onset of apoptosis due to hypericin (Buytaert et al., 2006b), though it did not prevent autophagy-associated cell death. In another case over-expression of Bcl-XL was reported as inducing resistance to apoptosis; however, cells remained sensitive to necrosis (Lavie et al., 1999). It is therefore probable that the increased expression of proteins from the Bcl-2 family, often found in about half of different human cancers (Reed, 1998), could impose a certain resistance to HY-PDT-induced apoptosis and switch the balance towards necrosis in some cell types (Agostinis et al., 2002). On the other hand, introduction of Bcl-2 into HT-29 cells led to stimulation of caspase-3 activation and a partial switch from necrotic to apoptotic cell death (Mikes et al., 2007). These and many other experiments prove that cell death induced by HY-PDT as well as its efficiency is significantly determined at the cellular level.

Although cells sensitized by activated hypericin show all the elementary hallmarks of apoptosis, recent studies have revealed that cell death may proceed via both caspase-dependent or -independent pathways. Initial experiments linked hypericin induced apoptosis with inhibition of protein kinase c (PKC) (Couldwell et al., 1994); however later, inhibition of PKC proved to be insufficient to cause apoptosis (Weller et al., 1997). Gradually more and more data are being accumulated, and lately hypericin-mediated cell death has proved to be initially triggered by an ER Ca^{2+} store emptying as a consequence of a sarco(endo)plasmic-reticulum Ca^{2+}-ATPase (SERCA2) protein level loss initiated by ER-associating hypericin irradiation (Buytaert et al., 2006a). Also in this case, apoptosis initiation required Bax and Bak, and their absence led to autophagy as claimed by these authors in another experiment, as already mentioned above (Buytaert et al., 2006b).

Moreover, hypericin also activates rescuing responses, chiefly governed by the activation of p38 MAPK (Assefa et al., 1999; Hendrickx et al., 2003). A more recent study showed that inhibition of p38-α and -β isoforms or p38 MAPK silencing in bladder cancer repressed the important subset of PDT-induced genes involved in inflammation, growth and invasion as well as antioxidant defense mechanisms. It also seems that the tumorigenic potential of cells surviving PDT with hypericin is governed by genes under control of the p38 MAPK pathway (Buytaert et al., 2007).

One of the key players in this “deadly” game is a protein with a very well-established role in cell death signalling known as p53. The crucial role of this protein and its signalling pathway has also been studied in the context of the PDT. The p53 protein is known to promote apoptosis via transcription-independent mechanisms as well as transcriptional activation of pro-apoptotic (Bax) (Zacal et al., 2005) or repression of anti-apoptotic genes (Weller, 1998). Still there is a lot of confusion about the role of p53 and its status in the efficacy of PDT. This is mostly due to the fact that PDT is generally considered non-

genotoxic, because the photosensitizers employed in PDT do not localize to the nucleus (Dougherty et al., 1998). Moreover, even the same authors puzzled over this question themselves (Fisher et al., 1998; Fisher et al., 1999) by introducing two separate works with contradictory results. It is evident that the question on the role of p53 in PDT does not have an easy and straightforward answer.

In an attempt to shed more light on this issue, we recently performed experiments with HCT-116 cells (Mikes et al., 2009). Concurrently with our experiments, another work by Lee and colleagues was published (Lee et al., 2006). In concert with their results we also concluded that p53 does not affect the ability of photo-activated hypericin to kill tumor cells. It does not affect the accumulation of cells in the G_2/M-phase either (Lee et al., 2006; Mikes et al., 2009). However, analysis of apoptosis in the final stages revealed suppression of its incidence in p53 knock-out cells, although interestingly, analysis of $\Delta\Psi m$ and loosening of cell membrane structure 24 h after PDT indicated greater susceptibility of the p53 knock-out cells (Mikes et al., 2009). Suppression of apoptosis through lack of p53 expression is, however, in good correlation with the work of Heinzelmann-Schwarz and colleagues (Heinzelmann-Schwarz et al., 2003). Summing up, the photocytotoxicity of HY-PDT seems to be independent of p53 status. Different conclusions are probably the result of different experimental approaches used for apoptosis detection, either in the initial phases of programmed cell death (Lee et al., 2006) or in the final stages of apoptosis (Heinzelmann-Schwarz et al., 2003). Taking the question on the role of p53 further, we also investigated long-term survival of p53-null *versus* wt-p53-expressing HCT-116 cells after HY-PDT under normoxic and hypoxic conditions, and we demonstrated that despite insignificant impact on overall toxicity or Bcl-2 levels, expression of p53 led to induced Mcl-1 as well as Bax levels, and it also suppressed the clonogenic efficiency of HCT-116 cells, especially in hypoxia.

Nowadays, increasing evidence supports the existence of caspase-independent cell death pathways that induce cell death in a manner that is as well-controlled and programmed as in apoptosis (Zong and Thompson, 2006). Caspase-independent cell death can therefore provide a backup suicide mechanism if the classical apoptosis machinery fails (Fiers et al., 1999; Leist and Jaattela, 2001). Recently, it has become clearer that necrotic cell death might also be as well-controlled and programmed as caspase-dependent apoptosis, and that it may be an important cell death mode that is both pathologically and physiologically relevant.

Necrosis has long been described as a consequence of physical or chemical stress, and therefore accidental and uncontrolled. But since PDT is a multifarious therapeutic approach, the shift from apoptotic to necrotic cell death can be set, when using a particular dye, by increasing the intensity of the PDT dose either by increasing the light dose or the concentration of the dye. Subsequent massive induction of ROS can lead to an immediate bio-energetic catastrophe, a drastic drop in ATP levels and general metabolic inhibition. But in contrast to necrosis caused by very extreme conditions, PDT is capable of inducing necrosis with the attributes of a normal physiological and regulated/programmed event. In fact, it is a major cell death morphology induced by PDT with compounds localized to the plasma membrane (reviewed in: Agostinis et al., 2004 and Almeida et al., 2004). This is most likely due to a rapid loss of plasma membrane integrity, incapability of maintaining ion fluxes across the plasma membrane, and fast depletion of intracellular ATP following photosensitization (Hsieh et al., 2003). The characteristic hallmarks of necrosis such as cytoplasmic swelling, irreversible plasma membrane damage, organelle breakdown (Grooten et al., 1993; Vercammen et al., 1998) as well as random degradation of DNA (in contrast to

apoptosis), which is revealed by DNA smear (Higuchi, 2003), are also important for its physiological function. In necrosis, the cellular contents leak into the extracellular environment, where they may act as a "danger signal", and consequently necrosis is usually associated with inflammation (Proskuryakov et al., 2003). In fact though, this process involves more specific mechanisms, since exposure of macrophages to necrotic cells is not sufficient to trigger macrophage activation and the concomitant induction of pro-inflammatory cytokine expression (Brouckaert et al., 2004; Cocco and Ucker, 2001). Rather, necrotic cells trigger an increase in the secretion of pro-inflammatory cytokines from independently-activated macrophages (Cocco and Ucker, 2001). Moreover, secretion of pro-inflammatory cytokine IL-6 by necrotic and not by apoptotic cells (Vanden Berghe et al., 2006) produces further evidence of a controlled pro-inflammatory mechanism activated by necrotic cells.

When referring to necrosis as a programmed cell death mode, the denotation "necroptosis" is also being used to make it distinct from uncontrolled accidental death. Moreover, as this denotation is relatively new, necrotic cell death identified and presented in some papers might in fact be necroptosis. But when it is not specifically identified by biochemical pathways it cannot be proved. Some discrepancy can therefore be found in the literature.

Necrotic cell death is not the result of one well-described signalling cascade, but is the consequence of extensive crosstalk between several biochemical and molecular events at different cellular levels. Recent data indicate that serine/threonine kinases RIP3 and RIP1 (receptor interacting protein) may act as central initiators. RIP3 appears especially crucial since cells that do not express it are defective for necroptosis induction upon TNF treatment (He et al., 2009; Zhang et al., 2009). Zhang and colleagues suggested that the role of RIP3 in apoptosis/necroptosis switching should at least partly occur through increasing energy metabolism-associated ROS production (Zhang et al., 2009). Evidence for involvement of RIP3/RIP1 in necroptosis invoked by HY-PDT is lacking up to date, although it has been demonstrated that in some cellular models necrosis might be the preferred mode of death (Mikes et al., 2007). However, in the case of 5-ALA-PDT-induced cell death, necroptosis was found to be dependent on RIP3, which forms aggregates and forms a pro-necrotic complex with RIP1 following photosensitization (Coupienne et al., 2011b).

Calcium and ROS are the main players during the propagation and the execution phases of necrotic cell death, directly or indirectly provoking damage to proteins, lipids and DNA, which culminates in disruption of organelle and cell integrity (Festjens et al., 2006).

The central role of Ca^{2+} in photo-oxidative initiation of necrotic cell death of neuronal and glial cells by photosensitizers, in a mixture of different sulfonated aluminum phthalocyanines (AlPcSn), has been reported to involve calmodulin and calmodulin-dependent kinase II (CaMKII) signalling (Uzdensky et al., 2007). In an epithelial breast tumor cell line the mode of cell death was found to be site-specific with respect to the distribution of a fixed level of protoporphyrin IX (PpIX). Apoptosis was the preferred mode of photokilling when PpIX was associated to the mitochondria, until this pathway was outmatched by necrosis, occurring in the absence of cytochrome c release and caspase signalling, when most of the porphyrin diffused to other cellular targets (Kriska et al., 2005). Some cationic porphyrins, which have been shown to relocalize from the plasma membrane to the cytosol during irradiation, have been reported to cause photoinactivation of procaspase-9 and procaspase-3 (Kessel, 2002). Since inhibition of caspases has been documented as a signal shifting the

mode of cell death in favor of necrosis, this photosensitizer-mediated caspase inactivation mechanism in the cytosol could result in the activation and propagation of a necrotic cell death pathway.

With regard to necrosis, some authors believe that unravelling the signalling cascades contributing to necrotic cell death might help to develop tools to specifically interfere with necrosis at certain levels of signalling. Inasmuch as necrosis occurs in both physiological and pathophysiological processes, and is capable of killing tumor cells that have developed strategies to evade apoptosis, more detailed knowledge of necrosis may be exploited in therapeutic strategies (Festjens et al., 2006).

Autophagy

Autophagy (Greek "self eating") is not currently considered to be primarily a cell death program. It is a major catabolic process in eukaryotic cells intended for recycling of cytoplasmic components and maintaining survival by removing damaged organelles, toxic metabolites or intracellular pathogens (Levine and Klionsky, 2004). Autophagy may also promote cell death through excessive self-digestion and degradation of essential cellular constituents (Debnath et al., 2005; Gozuacik and Kimchi, 2004). Although many unanswered questions remain concerning the molecular players of "autophagic cell death", this cellular program is thought to proceed in the absence of caspase-signalling or even to be activated under conditions of caspase inhibition (Shimizu et al., 2004).

The initial step in autophagy is the formation of a double membrane structure that sequesters cytoplasmic components as well as organelles and shapes the autophagic vacuoles or the so-called autophagosomes (Levine and Klionsky, 2004). Eventually, these autophagosomes fuse with lysosomes and their cytoplasmic material is degraded by lysosomal hydrolases. A family of autophagy-related genes (Atg) discovered in yeast and almost integrally conserved in all eukaryotic phyla, controls the formation of autophagosomes. Autophagy is regulated by class I and class III phosphatidylinositol 3-kinase (PI3K) signalling pathways, which have been reported to inhibit and stimulate autophagy, respectively (Petiot et al., 2000). Formation and completion of the sequestering vesicle is a complex mechanism which is under positive control of the class III PI3K complex, and involves several autophagy regulators including Atg6/Beclin 1 and Atg8/LC 3 (Levine and Klionsky, 2004).

The functional contribution of this catabolic process in cell death is still uncertain, as it is currently unclear whether autophagy directly contributes to cell death or is a failed effort to preserve cell viability. However, recent studies suggest that autophagy may regulate cancer development and progression as well as being a response to cytotoxic therapy (Gozuacik and Kimchi, 2004; Kondo et al., 2005). Several anticancer agents including tamoxifen, rapamycin, arsenic trioxide, temozolomide, histone deacetylase inhibitors, ionizing radiation, vitamin D analogue and etoposide have been reported to kill cancer cells and concurrently induce autophagy as part of a stress response (Kondo et al., 2005). There are, however, unresolved questions on the molecular mechanisms utilized by these drugs to trigger autophagy in cancer cells and the direct implication of autophagic cell death following anticancer treatment.

Recent studies have shown that PDT may induce non-apoptotic cell death associated with the induction of autophagy (Buytaert et al., 2006a; Kessel et al., 2006). Due to the high reactivity of photogenerated ROS it is not surprising that autophagy is initiated in an attempt to remove heavily-damaged organelles or to degrade large aggregates of cross-linked proteins produced by photochemical reactions, which cannot be removed by the ubiquitin–proteasome system or by the degradation associated with ER. Since autophagy is a self-limiting process, its persistence is liable to result in metabolic and bioenergetic collapse, which is causative for cell death (Buytaert et al., 2006b). Interestingly, it has been demonstrated lately that autophagy-related signalling pathways activated in response to PDT contribute to cell resistance (Davids et al., 2009; Dewaele et al., 2011).

Alternatively, the function of autophagy could be orchestrated by dedicated signalling molecules and switched from a survival to a lethal pathway in certain instances. Cell death modalities in HY-PDT have also been examined in wild-type murine embryonal fibroblast (MEFs) and in apoptosis-deficient Bax−/−Bak−/− MEFs. In these cells, all the biochemical hallmarks of apoptosis are prevented while photokilling continues unaltered through the induction of a non-apoptotic cell death pathway associated with ultrastructural and biochemical features of autophagy (Buytaert et al., 2006a). The pharmacological blockade of autophagy by the PI3K class III inhibitor wortmannin in apoptosis-deficient double knockout cells results in a significant reduction of cell death. These results are evidence that PDT can simulate an autophagic cell death pathway, at least under conditions of apoptosis inhibition. Further studies have shown that mitochondrial apoptosis and autophagy are concurrently promoted downstream of ER damage in PDT-treated cells (Buytaert et al., 2007; Kessel et al., 2006). The simultaneous induction of autophagy and apoptosis has been reported in murine leukemia L1210 cells after PDT with 9-capronyloxytetrakis (methoxyethyl) porphycene (CPO) (Kessel et al., 2006; Kessel, 2006). Interestingly, protection of CPO-mediated Bcl-2 photodamage reduced not only caspase activation but also the conversion of LC-3 I into the lipidated LC-3 II form, a biochemical hallmark of autophagy (Mizushima, 2004). This led to the suggestion that loss of native Bcl-2 may regulate both apoptotic and autophagic pathways following CPO-mediated PDT (Kessel, 2006). This hypothesis is based on the observation that Bcl-2 and/or Bcl-XL can inhibit not only apoptosis but also Beclin 1-dependent autophagy and Beclin 1-dependent autophagic cell death (Pattingre et al., 2005) through a direct interaction which has been recently shown to require the BH3 domain in Beclin 1 (Oberstein et al., 2007). However, the fact that the concomitant engagement of both apoptosis and autophagy occurs also in HeLa cells following HY-PDT, regardless of the reduction in the expression levels of anti-apoptotic Bcl-2 proteins, suggests that other molecular determinants can play a key role in this process (Buytaert et al., 2007).

The specific ROS-damaged subcellular site or organelle and the cargo of the autophagic vacuole are factors which could potentially influence the outcome of the autophagic process in PDT-treated cells. For instance, depending on the type and degree of organelle dysfunction, a degradative pathway targeting preferentially the photo-oxidized organelle or the cross-linked protein aggregates for autophagy could be specifically initiated. Examples of specific degradation pathways for damaged mitochondria, a process called mitophagy, have been documented in cultured hepatocytes (Elmore et al., 2001). This mitophagy often precedes apoptosis; however, this process is hardly noticeable without caspase inhibition due to rapid cell demise during apoptosis (Kundu and Thompson, 2005). Whether it is a process leading to delayed cell death by preventing leaking mitochondria from spilling out their pro-apoptotic

proteins and from generating ROS, or it is supposed to accelerate cell demise by degrading the major contributors of cellular ATP production, needs to be clarified (Kundu and Thompson, 2005). In situations where the ER is the main photo-damaged organelle and the mitochondria are spared from major alterations as reported in Bax-deficient cells (Buytaert et al., 2006a; Chiu et al., 2005), autophagy could target the ER for extensive engulfment and degradation resulting in the activation of a cell death pathway through a molecular mechanism, which still needs to be clarified (Buytaert et al., 2006a).

Cross-talk between autophagy and other modes of cell death is still not evident, and therefore is being studied intensively. However, it has been shown that introduction of RIP3 into RIP3-deficient cells surprisingly stimulated apoptosis and autophagy at the expense of necrosis in response to 5-ALA-PDT. The researchers claimed that in this context, autophagy represents a protective mechanism against cell death induced by PDT (Coupienne et al., 2011a). It is likely therefore that autophagy may contribute to cancer cell survival and negatively interact with the therapeutic outcome. On the other hand, pharmacological control of autophagic processes could potentially contribute to therapeutic efficiency.

The idea that cellular attributes are significantly involved in overall cytotoxicity is also confirmed by the rise of resistance in response to the photocytotoxic insult of HY-PDT. It has been demonstrated that fractionation of light doses may induce cellular resistance resulting in better cell survival linked with changes in the cell cycle, involving reduced hypericin content, decreased reactive oxygen species production, suppressed phosphatidylserine externalization, as well as altered expression of HSP70, GRP94, clusterin, nuclear factor NF-κB, IκB-α or Mcl-1 (Kulikova et al., 2011).

In addition to causing multi-drug resistance, ABC transporters have great impact on the pharmacokinetics of the different photosensitizers. Among the ABC transporters identified so far, three members of the ABC transporter family, P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP), appear to play an important role in the development of MDR in cancer cells exposed to PDT. BCRP is one of the crucial factors that affects the efficacy of PDT by extruding a wide variety of photosensitizers, especially porphyrins, for example protoporphyrin IX (Bebes et al., 2011; Jonker et al., 2002; Liu et al., 2007; Ogino et al., 2011; Robey et al., 2005), hematoporphyrin (An et al., 2009; Tamura et al., 2006; Tamura et al., 2007), pheophorbide a (An et al., 2009; Jonker et al., 2002; Robey et al., 2004; Robey et al., 2005; Tamura et al., 2006; Tamura et al., 2007) and pyropheophorbide and methylester (Robey et al., 2005). Besides the porphyrins, BCRP has been found to affect also the efficacy of PDT mediated by chlorin e6 (Robey et al., 2005), Photochlor, Verteporfin (Liu et al., 2007) and Photofrin (Usuda et al., 2010). Regarding the relationship between photosensitizers and P-gp activity, controversial results have been reported.

Depending on the P-gp phenotype, no differences were found in meta-tetra (hydroxyphenyl) chlorin and phthalocyanine derivates accumulation (Frazier et al., 1993; Teiten et al., 2001). On the other hand, decreased production of protoporphyrin IX was detected in MCF-7/ADR cells as compared to parental MCF-7 cells (Tsai et al., 2004) and in P-gp expressing human uterine sarcoma cells (Chu et al., 2008). Tang and colleagues demonstrated that intracellular uptake of pheophorbide a was not reduced by P-gp expression (Tang et al., 2009). Moreover, pheophorbide a-mediated PDT down-regulated the expression of P-gp and inhibited the growth of hepatoma xenograft tumor (Tang et al., 2009). HY itself or HY-PDT in the context of functional and expressional upregulations of ABC transporters

revealed induction of MRP1 and BCRP, as well as suggesting that HY itself might be a substrate molecule for these transporting systems (Jendzelovsky et al., 2009).

Although induction of resistance is a serious complication, targeting of specific pathways may balance it. For example HY-PDT has proved to be effective against HER2-positive cancer cell lines specifically acting against HER2 protein via destabilization of HSP90 chaperone protein, with even more pronounced changes with specific degradation of HER2 (Koval et al., 2010).

Table 2. An example of effects induced by HY-PDT

Effect	Details	References
Antimicrobial effects	effective against Gram positive methicillin-sensitive and methicillin-resistant Staphylococcus aureus, but ineffective against Escherichia coli	(Yow et al., 2012)
Antiviral	hypericin inhibits binding and entry of HIV virus into T-lymphocytes	(Acosta and Fletcher, 1994)
Damage associated molecular patterns (DAMPs)	exposure of the HSP-70 and calreticulin molecules on the cell surface	(Garg et al., 2012)
hypericin accumulation	lower efficiency of topical HY-PDT in comparison to PDT with methyl-aminolevulinic acid (Me-ALA) when applied on mouse skin with actinic keratinosis	(Boiy et al., 2011)
	higher hypericin incorporation does not have to correlate with photocytotoxicity	(Mikes et al., 2011)
autophagy induction	autophagic response occurrs in cells with photodamaged lysosomes	(Reiners et al., 2010)
HER2 degradation	HY-PDT invokes HER2 degradation	(Koval et al., 2010)
p53	indepedence of direct toxicity from p53 expression	(Lee et al., 2006; Mikes et al., 2009)
inhibition of superoxide in neutrophils	inhibition of tyrosine kinase, PKC and NADPH oxidase in neutrophils is responsible for light-dependent inhibition of superoxide	(Nishiuchi et al., 1995)
p44ERK1	light dependent inhibition	(Assefa et al., 1999)
p42ERK2	light dependent inhibition	(Assefa et al., 1999)
hemoglobin	HY-PDT changes to structure of hemoglobin and enhances its catalytic activity	(Zhao et al., 2008)
induction of angiogenic proteins	induction of VEGF, TNF-α, IFN-α, bFGF in cellular targeting HY-PDT	(Bhuvaneswari et al., 2008)
VEGF induction	vascular damage after PDT stimulates VEGF production	(Bhuvaneswari et al., 2007a)
down-regulation of adhesion molecules	cadherin 5, collagen alpha 1 and 3	(Bhuvaneswari et al., 2008)
JNK1 activation	resistance to apoptosis	(Assefa et al., 1999)
p38 MAPK activation	resistance to apoptosis	(Assefa et al., 1999)
IL-6 induction	induction of IL-6 in poorly differentiated cells in comparison to differentiated cells after HY-PDT	(Du et al., 2006a)
p38α MAPK inhibition	blocks VEGF and suppresses tumor promoted endothelial cells migration	(Hendrickx et al., 2005)
COX-2 upregulation	mediated by p38α/β MAPK that stabilizes the COX-2 mRNA transcript	(Hendrickx et al., 2003)

It has also been demonstrated *in vitro* that pre-treatment of tumor cells with various inhibitors of arachidonic acid metabolism (Kleban et al., 2006; Kleban et al., 2007; Kleban et al., 2008), ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) (Kello et al., 2010), or genistein (Ferenc et al., 2010) can improve the efficacy of HY-PDT.

Anti-Vascular Effect of PDT with Hypericin

As already mentioned, targeting of tumor vasculature appears to be a promising approach to tumor therapy. The anti-vascular potential of HY-PDT was introduced in the study by Chen and colleagues, where higher efficacy correlated with plasma concentration rather than tumor drug level (Chen et al., 2001). Neither tumor hypoxia nor increasing tumor oxygenation could significantly affect the effectiveness of various PDT protocols.

These results suggested tumor vasculature damage as the potential primary mechanism of HY-PDT (Chen et al., 2001). Similarly, results demonstrating higher relative regression percentage of treated tumors compared to cellular-targeted PDT have been presented (Olivo and Chin, 2006). Interruption of the nutrition or oxygen supply and subsequent malnutrition may have a more global effect on a tumor, although oxygen in particular is crucial for the photocytotoxic action of PDT, and so it might limit effects to the cellular level. And as a short drug/light interval-mediated PDT primarily based on the differential uptake of the photosensitizer into tumor-associated vasculature as opposed to the cellular compartments of the tumor itself (Olivo and Chin, 2006) are regularly used, the intracellular level of photosensitizer in this schema is logically much lower than needed to make any significant impact on tumor cells.

In line with these facts Cavarga and colleagues presented another approach based on fractionated dosing of HY. They proved that HY administrated 1 and 6 hours before irradiation can produce a better therapeutic response than any single administration. Their results suggest that cellular and vascular toxicity (and perhaps immune response) contribute to the overall therapeutic outcome (Cavarga et al., 2005).

On the other hand, even though the destruction of tumor vasculature might be an effective modality in treatment, stress-related release of angiogenic growth factors and cytokines as a consequence of the hypoxic condition within tumors could possibly diminish the efficacy of PDT by promoting tumor regrowth (Bhuvaneswari et al., 2007a). Avastin (bevacizumab), a vascular endothelial growth factor (VEGF) specific monoclonal antibody in combination with chemotherapy is already offering hope to patients with metastatic colorectal cancer. Bhuvaneswari and colleagues evaluated the combination of HY-PDT and Avastin on VEGF levels as well as its effect on overall tumor response of bladder carcinoma xenografts established subcutaneously in Balb/c nude mice (Bhuvaneswari et al., 2007b). The results demonstrated improvement in tumor responsiveness to therapy by Avastin along with PDT. The expression of VEGF in tumors was minimal.

Angiogenic proteins such as angiogenin, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and interleukins (IL-6 and IL-8) were also found to be downregulated in groups treated with combination therapy (Bhuvaneswari et al., 2007b). Similar results were achieved by the same authors in nasopharyngeal carcinoma xenografts with another angiogenesis inhibitor, Celebrex (Bhuvaneswari et al., 2007a).

Light-Independent Action of Hypericin (Hypericin on the "Dark Side")

Besides the light-dependent photodynamic action of HY *in vivo*, some anti-metastatic action in the dark has been recorded as well (Blank et al., 2004). In this experiment, the administration of HY in 6 fractionated doses increased the survival rate in mice bearing primary breast adenocarcinoma (DA3) or squamous cell carcinoma (SQ2) tumors, although all animals eventually died during the experiment. HY administration in up to 6 fractionated doses after surgery led to significant improvement in long-term survival (evaluated up to 300 days after tumor inoculation). Thus, in the absence of photosensitization, HY is unlikely to provide effective protection against primary tumors; however, besides its photodynamic action HY may serve as an anti-metastatic agent. Histological evaluation of lung metastases revealed massive destruction 72 hours after HY treatment, but involvement of the immune system was excluded since no anti-tumor protective activity had been observed in animals previously cured with HY (Blank et al., 2004). The authors claim that most experiments proving absence of HY cytotoxicity in the dark limited the exposure of cells to a short time of a few hours. Indeed, Blank and colleagues applied up to 6 HY administrations at 5-day intervals and the therapeutic results were in positive correlation with the number of repeated administrations (Blank et al., 2001). *In vitro* experiments have established the mechanism of HY cytotoxic action in the dark as an enhanced heat-shock protein 90 (HSP90) ubiquitinylation and consequent destabilization of its client proteins including Raf-1. The decline in Raf-1 prevents downstream activation of extracellular signal-regulated kinase 1/2 kinases, inhibits the Ras/Raf pathway and leads to tumor cell proliferation arrest. Events such as cell-cycle arrest in the G_2/M-phase, increased cell volume and multinucleolation observed in these experiments are hallmarks of mitotic cell death (Hwang et al., 2001; Blank et al., 2003).

Another mechanism of HY action in the dark was suggested by Martinez-Poveda and colleagues. They discovered that HY in the dark is able to inhibit key steps of angiogenesis including endothelial cell proliferation, tubular formation on Matrigel, extracellular matrix degradation by urokinase, migration and invasion (Martinez-Poveda et al., 2005).

On the other hand, we have also demonstrated that HY in dark conditions is also capable of inducing expression of ABC-transporting systems, which are often linked with the MDR-phenotype (Jendzelovsky et al., 2009).

Summing up the submitted data, we can predict that both cellular and vascular effects possibly play their role in the "dark action" of HY *in vivo*.

Interaction with Enzyme Activity and Expression

Another mechanism involved in HY cytotoxicity and photocytotoxicity could be the interaction with the activity and expression of enzymes. As mentioned above, HY and other constituents of St. John's wort are known to inhibit enzymatic activity of proteins and even interact with their expression. A broad list of HY inhibitory actions against different enzymes involved in cell signalling as well as in management of depression can be found in the review by Kubin and colleagues (Kubin et al., 2005). Of those enzymes which are known to play a

significant role in tumorigenesis, survival and proliferation regulation, HY inhibits phosphatidylinositol 3-kinase (PI3K), PKCs, protein tyrosine kinase activities (PTK) of EGFR and the insulin receptor, at the same time remaining ineffective towards the cytosolic protein tyrosine kinases. But photosensitized inhibition by HY is not restricted to receptor-PTKs, since the Ser/Thr protein kinases (protein kinase CK-2 or mitogen-activated kinase) are also extremely sensitive to inhibition (IC50s already in nM concentrations). Inhibition of some of these enzymes proved to be irreversible after irradiation (Agostinis et al., 1995).

Inhibition of PKCs (Agostinis et al., 1995) or PTKs (de Witte et al., 1993) (among others) measured as IC50 of their enzymatic activity proved to be light-dependent. Utsumi and colleagues even demonstrated light-intensity-dependent inhibition of PKC by HY, whereas minimal action was detected under dark conditions (Utsumi et al., 1995b). Likewise structure-dependent selectivity of PKCs to HY was also confirmed by light-dependent inhibition with calphostin (a perylenequinone with structure and properties similar to HY) (Bruns et al., 1991). Specificity of HY inhibitory action, on the other hand, is underlined by the fact that it is largely inactive against a group of cytosolic protein tyrosine kinases Lyn, Fgr, TPK-IIB and c-Src kinase (Agostinis et al., 1995).

Since the photodynamic action of HY is based on induction of oxidative stress, the *in vivo* inhibition of antioxidant enzymes by HY itself is also interesting. HY inhibition of glutathione reductase, selenium-dependent glutathione peroxidase, glutathione S-transferase and superoxide dismutase was compared by Johnson and Pardini in the dark and after light irradiation (Johnson and Pardini, 1998). The most efficient was the inhibition of glutathione reductase and moreover it proved to be light-independent with IC50 of about 2 nM for both dark and light irradiation (Johnson and Pardini, 1998). The inhibition of the other antioxidant proteins tested in this study proved to be IC50 efficient in micromolar concentrations and light-dependent.

To understand the mechanism of HY light-dependent inhibitory action we can refer to the work of Thomas and colleagues, who documented a positive relationship between mitochondrial succinoxidase inhibition and generation of singlet oxygen by HY-PDT (Thomas et al., 1992). These results suggest the involvement of type II oxidative mechanism of HY inhibitory action, at least against succinoxidase, although they might have more general implications as well.

Evaluation of St. John's wort for inhibitory effects towards human cytochromes P450 (CYP) has revealed possible interactions of its constituents. Since CYPs represent a large family of proteins involved in the metabolism of drugs and other xenobiotics as well as some endogenous substrates, usage of herbal products such as St. John's wort is considered as risky due to potential pharmacokinetic drug interactions *in vivo* (Obach, 2000). The effects of particular constituents in St. John's wort differed, but HY *per se* proved to be a competitive inhibitor of CYP2C9, CYP2D6 and CYP3A4, with IC50 below 10 μM (Obach, 2000).

As widely reviewed by Pal and Mitra (Pal and Mitra, 2006), HY together with other constituents of St. John's wort significantly interact with the activity and expression of P-glycoprotein and CYP3A4, though some of their effects are similar as well as contradictory, and also depend on exposure time. Acting together, P-gp as an efflux pump and CYP3A4 are capable of metabolizing about 50% of drugs used today. They reduced the bioavailability of drugs and therefore participate significantly in the multidrug resistance phenotype of many tumors.

Pure preparations of HY inhibited the efflux of ritonavir by P-gp and inhibited CYP3A4-mediated cortisol metabolism (Patel et al., 2004). However, prolonged exposure decreased CYP3A4 mRNA on one hand, but it increased the mRNA level of P-gp on the other (Patel et al., 2004). Other experiments have confirmed that HY, unlike hyperforin, has no impact on the steroid and xenobiotic receptor (SXR) (Wentworth et al., 2000), which induces the expression of CYP3A4. Nevertheless, most studies report the effect of crude extracts of St. John's wort, and therefore further studies with pure HY and other extract constituents are necessary to specify potential interactions with different therapeutic protocols (Pal and Mitra, 2006).

Strategies to Overcome the Resistance to HY-PDT

Research focused on resistance to cancer treatment as well as clinical praxis has already proved that specific combinations of various therapeutic modalities may help to overcome or suppress complications with resistance to therapy. Consequently, there is a logical assumption that the efficacy of HY-PDT might be improved through combinations with other therapeutic modalities. As the group of nonsteroidal anti-inflammatory drugs (NSAIDs) has been found to demonstrate chemopreventive action against various tumors, which was first linked mostly with the modulation of arachidonic acid (AA) metabolism, we decided to carry out extensive screening of various inhibitors of cyclooxygenase (COX; diclofenac, flurbiprofen, ibuprofen, indomethacin, SC-560 and rofecoxib), lipoxygenase (LOX; nordihydroguaiaretic acid, esculetin, AA-861, MK-886 and baicalein) and P450-monooxygenase pathways (proadifen) of the AA metabolism (Kleban et al., 2007). Whereas COX-1 (SC-560) and COX-2 (rofecoxib) specific inhibitors proved to be ineffective or even protective, nonspecific COX inhibitors, especially flurbiprofen, ibuprofen and indomethacin, demonstrated the ability to effectively stimulate the action of HY-PDT, as nicely proved by intensive suppression of cell viability and increased numbers of floating cells. The LOX inhibitors showed contradictory effects as well. Whereas nordihydroguaiaretic acid and AA-861 showed a more or less antagonistic effect against HY-PDT action and stimulated cell survival as well as global metabolic activity of the whole population (MTT assay), MK-886 and baicalein were able to stimulate the efficiency of HY-PDT. The action of esculetin produced variable responses with regard to its concentration. The action of proadifen as the inhibitor of P450 monooxygenase also proved to be effective and stimulated the photocytotoxic action of HY (Kleban et al., 2007). Moreover, in another subsequent study we proved that proadifen enhances HY accumulation in cells through suppression of ABC-transporting systems activity (Jendzelovsky et al., 2009).

As MK-886 has been found to be one of the most effective inhibitors capable of stimulating the efficiency of HY-PDT (also depending on effective concentrations), we focused closer on the mechanisms behind its cytotoxic action (Kleban et al., 2006; Kleban et al., 2008). When applied alone, MK-886 induced necrosis as determined by morphological analysis, but when applied as pre-treatment to HY-PDT, apoptosis was induced in addition, as also proved by specific cleavage of PARP protein. The mutual effect also corresponded with an increased percentage of cells with dissipated mitochondrial membrane potential and significant changes in cell-cycle progression leading to accumulation in the S-phase at lower

concentrations and G1/G0-phase accumulation at 15 μM of MK-886 (Kleban et al., 2006). G1/G0-arrest was accompanied with increase in cyclin E level and decrease in cyclin A, cdk-2 and pRb expression, indicating blockage of G1/S transition. Programmed cell death initiation was identified by increased activity of initiation (casp-8/-9) or execution (casp-3) caspases. Although pre-treatment with MK-886 did not increase ROS levels globally, it switched the ratio of superoxide/hydrogen peroxide at the expense of superoxide. The loss of clonogenic potential of cells treated with the combination of MK-886 and HY-PDT highlighted their mutual effects (Kleban et al., 2008).

A diet rich in some polyunsaturated fatty acids was found to suppress tumorigenesis as well (Karmali et al., 1989). Their combination with various cytotoxic compounds seems therefore to be a logical option. Indeed, this concept has proved to be efficient under *in vitro* conditions, showing enhanced action of HY-PDT on cells pre-treated with ω-3 (docosahexaenoic acid; DHA) and ω -6 (arachidonic acid; AA) fatty acids (Kello et al., 2010). Although HY is an amphiphilic molecule, applied PUFAs did not enhance its accumulation in cells, but they did stimulate pro-apoptotic events such as activation of casp-3 (DHA) and the percentage of cells with apoptotic morphology. Both PUFAs stimulated production of reactive species within the cells, demonstrated by intensive lipid peroxidation, but with preferential production of ROS by DHA and reactive nitrogen species (RNS) by AA (Kello et al., 2010).

Genistein is known as a naturally-occurring anticancer drug belonging among the isoflavones. It is a tyrosine kinase inhibitor capable of inducing apoptosis in tumor cells by itself (Yu et al., 2004). We have demonstrated in one of our previous works that mutual combination of genistein with HY-PDT led to suppression of Bcl-2 and of Akt and Erk1/2 phosphorylation, induced cell-cycle arrest in the G_2/M-phase, activated casp-7, PARP cleavage and also elevated the percentage of cells with typical apoptotical morphology. These changes corresponded with suppressed cell proliferation with clonogenic potential. All these events evoked improved efficiency of HY-PDT (Ferenc et al., 2010).

As HER2 protein overexpression is regularly found in approx. 25-30% of breast cancers and it is generally associated with poor prognosis (Slamon et al., 1989), the treatment in clinical praxis is regularly combined with monoclonal antibody against HER2 (herceptin), which improves the disease-free survival of patients with metastatic breast cancer (Nielsen et al., 2009). To demonstrate the potential of HY-PDT for clinical applications we tested the combination of HY-PDT with selective HER2 inhibitor (AG 825) and found that HY-PDT itself induces degradation of mRNA for HER2 (Solar et al., 2011a) and HER2 protein in time-dependent manner via targeting of HSP90 (Koval et al., 2010). This mechanism promotes degradation of HER2 in lysosomes. The combination of HY-PDT with AG 825 even enhanced the photocytotoxic action of HY and led to accumulation of cells in the S-phase of the cell cycle, enhanced mitochondrial membrane potential dissipation, suppression of Akt levels and phosphorylation, leading to total degradation of HER2, Mcl-1 and Bcl-xL as well as to inhibition of colony formation (Koval et al., 2010).

Recently we have shown that HY-PDT might be also enhanced by pre-treatment with selective farnesyltransferase inhibitor manumycin A, which induced apoptosis represented for example by activation of casp-3/7 and PARP cleavage, or suppressed clonogenic ability of the tumor cells (Sackova et al. 2011).

Table 3. List of therapeutic applications combining HY-PDT. The mutual combinations are considered synergistic (+), ineffective (0) or antagonistic (-)

Combination of HY-PDT with:	Details	+/0/-	References
phenylthiourea	reversible tyrosinase inhibitor that overcomes pigmented melanomas resistance to PDT	+	(Sharma et al., 2011)
AG 825	combination of HY-PDT with selective HER2 inhibitor AG 825 leads to total HER2 degradation	+	(Koval et al. 2010)
SB202190, SB203580	inhibitors of p38α/p38β MAPK isoforms enhance the HY-PDT	+	(Chan et al., 2009)
celecoxib (celebrex)	COX-2 and angiogenesis inhibitor decreases VEGF levels and enhances the overall outcome	+	(Bhuvaneswari et al., 2007a; Yee et al., 2005)
bevacizumab (avastin)	monoclonal antibody targeting VEGF, thus preventing angiogenesis	+	(Bhuvaneswari et al., 2010; Bhuvaneswari et al., 2007b)
manumycin A	selective farnesyltransferase inhibitor enhances HY-PDT efficiency	+	(Sackova et al., 2011)
SU6668	anti-angiogenic therapy with SU6668, a relatively broad spectrum RTK (receptor-associated tyrosine kinase) inhibitor for VEGF, FGF and PDGF receptors can increase HY-PDT efficacy	+	(Zhou et al., 2005)
5-aminolevulinic acid (5-ALA)	enhanced toxicity after white light irradiation linked with higher production of protoporphyrin IX (PpIX)	+	(Schneider-Yin et al., 2009)
genistein	tyrosine kinase inhibitor genistein downregulates Bcl-2, suppresses Akt and Erk1/2 phosphorylation	+	(Ferenc et al., 2010)
17-DMAG	blocks nucleotide binding of HSP90 and induces ubiquitination and proteosomal degradation of client proteins including HER2	+	(Solar et al., 2011b)
temozolomide	hypericin increases growth inhibitory potential of temozolomide	+	(Gupta et al., 2006)
PUFAs	docosahexaenoic acid (DHA) and arachidonic acid (AA) stimulated accumulation of reactive oxygen/nitrogen species and induces lipoperoxidation	+	(Kello et al., 2010)
proadifen	P450-monooxygenase inhibitor - affects function of MRP1 and BCRP and increases HY accumulation	+	(Jendzelovsky et al., 2009; Kleban et al., 2007)
mitoxantrone	HY used for release of mitoxantrone from intracellular vesicules by photochemical internalisation delivery method	+	(Adigbli et al., 2007)
nordihydroguaiaretic acid (NDGA)	lipoxygenase inhibitor	+/-	(Kleban et al., 2007)
esculetin	5,12-lipoxygenase inhibitor	+	(Kleban et al., 2007)
MK-886	5-lipoxygenase inhibitor stimulated superoxide accumulation	+	(Kleban et al., 2006; Kleban et al., 2007; Kleban et al., 2008)
AA-861	5-lipoxygenase inhibitor	0	(Kleban et al., 2007)
baicalein	12-lipoxygenase inhibitor	+	(Kleban et al., 2007)
diclofenac	COX inhibitor	+	(Kleban et al., 2007)
flurbiprofen	preferential COX-1 inhibitor	+	(Kleban et al., 2007)
ibuprofen	preferential COX-1 inhibitor	+	(Kleban et al., 2007)

Combination of HY-PDT with:	Details	+/0/-	References
indomethacin	preferential COX-1 inhibitor	+	(Kleban et al., 2007)
SC-560	COX-1 specific inhibitor	-	(Kleban et al., 2007)
rofecoxib	COX-2 specific inhibitor	-	(Kleban et al., 2007)
anti-HSA conjugate	conjugate of HY with monoclonal antibody against hepatocyte specific antigen	+	(Fadel et al., 2010)
polylactic acid	polymeric nanoparticle delivery system	+	(Zeisser-Labouebe et al., 2006; Zeisser-Labouebe et al., 2009)
polylactic-co-glycolic acid	polymeric nanoparticle delivery system	0	(Zeisser-Labouebe et al., 2006)
mitomycin C	decreases tumor cell survival and delayed tumor growth in combination with HY-PDT	+	(Chen et al., 2003a)
irinotecan	hypericin used as PKC inhibitor enhances irinotecan action	+	(Chen et al., 2003b)

On the other hand, the hypothesis of enhanced resistance of tumour cells treated with erythropoietin, which is routinely used in the treatment of anemia, caused *inter alia* by the myelosuppressive effects of chemotherapy, proved to be well grounded also with regard to HY-PDT for ovarian cancer cells (Solar et al., 2008). This resistance was reversible by treatment with the specific Janus kinase 2 inhibitor or with genistein, suggesting a role for the specific erythropoietin-induced Janus kinase 2/STAT signal transduction pathway in PDT resistance (Solar et al., 2008).

Conclusion

Summing up, we may say that HY-PDT is a highly-efficient therapeutic modality capable of inducing severe destruction of various cellular targets as well as massive cell death *in vitro* as well as *in vivo*. It is important to consider the concepts of programmed cell death that are different from apoptosis, the idea of programmed necrosis (Proskuryakov et al., 2003) and the overturn of the autophagic repair process into a programmed event that is possibly executable after irreparable photodamage to crucial cellular structures (Buytaert et al., 2006b; Buytaert et al., 2006a; Kessel et al., 2006). Excluding both low, non-toxic doses which may induce resistance as well as intensive PDT leading to massive cellular destruction and bioenergetic catastrophe that might be defined as "instant" necrosis, whether evoked by high HY concentration or light dose, we can conclude that photo-activated HY induces a wide spectrum of programmed cell death modes. Considering the data it is likely that HY is an effective inducer of apoptosis, but if that is somehow prevented, then programmed necrosis or autophagic cell death may take part in whole process. Fortunately, it seems that if factors such as oxygen repository, HY content and light dose are not limiting, PDT with HY represents a viable option in cancer therapy. It is evident though that the mode of cell death resulting from HY photocytotoxicity is affected by multiple pro- or anti-apoptotic signalling pathways.

The importance of the vascular mechanism in HY-PDT under *in vivo* conditions is undeniable, though the preference for vascular versus cellular targeting seems to be dependent upon the relative distribution of photosensitizers in each compartment, which is

governed by the photosensitizer's pharmacokinetic properties and can be effectively manipulated by adjusting the photosensitizer drug administration and light illumination interval (drug-light interval) during PDT treatment, or by modifying the photosensitizer molecular structure (Chen et al., 2006). Further studies focusing on various delivery systems and/or different protocols may therefore greatly contribute to the higher efficiency and improve the applicability of HY-PDT.

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Chapter 6

Effect of Liposomal Photosensitizer as Advanced Photodynamic Therapy

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Abstract

Photodynamic therapy (PDT) with a photosensitizer and laser irradiation has been shown to have some effects on early or superificial tumor. And, PDT has been used on some vascular diseases, such as arteriosclerosis or aging macular degeneration. However, there are some problems on adverse reaction including skin damage or photosensitivity by retained photosensitizer in normal site and on unstable active oxygen generated from photosensitizer.

Liposomes are closing vesicle composing of phospholipid and are the principal means of effectively using medicines and are broadly recognized as a drug delivery system. There are some studies on the breakthrough against those problems of PDT by liposomalization of the photosensitizer such as photofrin (PF). In a human gastric tumor inoculated mice, PF level in tumor in the liposomal PF (LPF) group was significantly higher level by 2.4-fold of that in the PF solution (PFsol) group, whereas the PF levels in the skin were almost equal. At irradiation after PFsol or LPF administration, the volume of necrotic tumor and the apoptotic index of the tumor were significantly higher in the LPF group than in the PFsol group. Namely, the liposomalization of the photosensitizer increased its tumor accumulation, with a resulting enhancement of the therapeutic effect of PDT. Furthermore, polyethyleneglycol modified LPF (PEG-LPF) siginificantly enhanced phototoxicity, compared to that of PFsol. The amount of singlet oxygen from PFsol and each liposome was PEG-LPF = LPF > PFsol. Thus, the phototoxicity of PEG-LPF was significantly higher than that of PFsol or LPF. It is expected that formation of a fixed aqueous layer on the liposomal membrane by PEG-modification physically changed it into the stable state of PEG-LPF.

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In examination of the liposomalization of coproporphyrin I (CPI) with hydrophobic property as novel photosensitizer, the CPI concentration in tumors was higher after PEG modified liposomes (PEG-CPI) injection than after CPI liposomes (Lipo-CPI) or CPI solution. Therefore, PEG-CPI was likely to increase blood circulation and achieve greater accumulation of CPI in the tumor. When loaded into tumor cells, photosensitizers generate singlet oxygen during laser irradiation, resulting in the induction of necrosis in the cells. The order of magnitude of CPI tumor cells uptake was PEG-CPI > Lipo-CPI > CPI solution. Thus, the PEG modification of Lipo-CPI improved its tumor cell uptake. The cytotoxicity of PEG-CPI was significantly greater than the other formulations, suggesting that the cytotoxicity reflected the CPI concentration in tumor cells.

In conclusion, liposomalization of photosensitizer and PEG modification of liposome was confirmed to be effective tool in PDT. It is expected that these liposomes advance clinical PDT in future.

1. Introduction

Photodynamic therapy (PDT) with a photosensitizer and laser irradiation has been established as a potent and less invasive treatment for early or superficial tumors, including gastrointestinal, bronchopulmonary, and gynecological neoplasms [1-7]. Furthermore, PDT has been used on some vascular diseases, such as arteriosclerosis or aging macular degeneration [8-11]. PDT shows a more specific effect on tumors than other laser therapies, because it damages only the cells containing a photosensitizer, which is preferentially absorbed by malignant cells. The photosensitizer self-associates to form aggregates in tumors and these aggregates cannot move out because tumors do not have lymphatics. Several investigators have shown lipoproteins (particularly low density lipoproteins) play a role in the transport of the photosensitiser [12].

Among various attempts to enhance the therapeutic efficacy of PDT, the specific accumulation of the photosensitizer in the tumor is expected to increase its clinical applications, since undesirable accumulation, especially in the skin, impairs the patients' quality of life (QOL), resulting in increased hospital stay.

Photofrin, a hematoporphyrin derivative activated with red light of 630 nm, has been approved and commercialized in European and Asian countries, as well as in North America [13]. Especially in Japan, PDT with photofrin has been supported by government medical insurance since 1996. The antitumor effect of PDT is triggered by the singlet oxygen generated from the photosensitizer under laser irradiation [14-16]. The difference in concentration of the photosensitizer makes it possible to damage tumor tissue with less damage to the surrounding normal tissues [17]. However, photofrin as a photosensitizer is a lipophilic agent, and was shown to be slowly metabolized in the body. Importantly, photofrin is retained in the skin and produces some adverse reactions, including skin damage or photosensitivity. The patient, after undergoing PDT, must live in a dark room for 6–8 weeks due to this adverse reaction, causing a decline in the QOL [17]. To improve therapeutic index by photosensitizer and increase of QOL of patients, it is necessary to decrease this adverse reaction or to increase the laser sensitivity in PDT.

Liposomes are used as models of cell membranes to examine drug permeability and as drug delivery system (DDS) carriers for antitumor agents and other medicines. Furthermore, liposomes have also recently been found to be safe, non-viral vectors in gene targeting.

However, liposomes are easily opsonized with serum proteins (opsonization), after which they are taken up by reticuloendothelial system (RES) cells such as liver and spleen [18-21]. It is known that polyethyleneglycol (PEG) modification of the liposomal surface leads to the formation of a fixed aqueous layer around the liposomes due to interaction between the PEG-polymer and water molecules, which prevents the attraction of opsonins. As a result, PEG-modified liposomes escape being by the RES cells, and hence have a prolonged circulation time [21-29]. Now, some antitumor agents containing liposomes have been used clinically and have been shown to be effective against Kaposi's sarcoma in patients with AIDS or recurrent ovarian cancer [30, 31] or deep-mycosis [32] in some countries. Thus, Liposome is a superior tool due to its increased activity and decreased adverse reactions.

2. Photofrin

2.1. Preparation of Liposomal Photofrin

Preferential accumulation of some photosensitizers in tumors has been demonstrated [4, 33, 34], but more specific accumulation and decrease of its adverse reactions are expected to increase the usefulness of PDT and QOL of patients. The development of DDS of photosensitizer may increase the advantages of PDT.

Liposomalization of phtosensitizers was examined in some previous studies. Liposomal benzoporphyrin derivative monoacid ring A (BPD) showed an enhanced therapeutic effect against Meth A sarcoma [35], and liposomal Hypocrellin A (HA) showed an enhanced effect against S-180 sarcoma [36]. It was reported that a high accumulation of HA in liposomes at 12 h after intravenous administration compared with HA in dimethylsulfoxide-solubilized saline without liposomalization. The accumulation in tumor was slightly higher with liposomal BPD than with aquenous BPD was reported [37]. The studies about liposomalization of photofrin were reported only by Jiang [38] although photofrin have been commonly applied for the clinical use. They reported that liposomal photofrin prepared by dipalmitoyldiphosphati-dylcholine (DPPC) was preferentially accumulated in brain tumor, compared with normal brain in the glioblastoma or glioma. The analysis of liposomal characterization was essential after preparation because of the difficulty of liposomalization of photofrin, but the liposomal character including the composition of phospholipids etc., the organ distribution including specificity of tumor accumulation, the therapeutic effect against human tumors, or adverse reactions have not been satisfactorily investigated.

There are many methods of liposome preparation. The difference of entrapped efficacy and stability among various compositions of liposomes or preparation methods were investigated and reported previously [21]. The liposomalization of photofrin was applied by Bangham method [39].

Dimyristoylphosphatidylcholine (DMPC) (100 μmol), cholesterol (100 μmol), dimyristoylphosphatidylglycerol (DMPG) (60 μmol), and 30 mg of photofrin (10 μmol) were dissolved in chloroform : methanol (4:1, v/v). After evaporation of the solvent, the thin lipid film was hydrated in a water bath at 50 – 60 °C with 8.0 ml of 10 mM phosphate buffer (pH 7.0) containing 260 mM sucrose. The resulting suspension was then sonicated for 20 min at above the phase transition temperature. The liposome suspension was then passed through

two stacked polycarbonate membrane filters with 0.2 μm pores, after which it was passed five times through filters with 0.1 μm pores, and a homogeneous liposome suspension was thus obtained. Liposome suspension was dialyzed in 10mM phosphate buffer (pH 7.0) containing 260 mM sucrose for 16 h. The entrapment rate of photofrin in liposome was about 80%, and the liposome with 106.2 ± 3.6 nm of diameter was stable for more than 3 months at room temperature.

2.2. Effect of Liposomal Photofrin on Human Gastric Tumor

2.2.1. Tissue Distribution of Photofrin

The liposomes consisted of DMPC, cholesterol, DMPG, have been demonstrated to have higher entrapped efficacy and stability than any other type of liposomes. Namely, entrapped ratio and stability of liposomal photofrin was shown to be satisfactory and its diameter was small enough for the passive targeting.

MT-2 human gastric cancer xenograft was transplanted onto the backs of nude mice. At day 28 after tumor implantation, photofrin solution or liposomal photofrin (10 mg/kg) was injected to these mice via a tail vein. MT-2 was poorly differentiated adenocarcinoma line. The photofrin concentration in the tumors increased until 8 h after liposomal photofrin injection (1.20 μg/g), followed by a decline over the course of the next 24 h. By contrast, the maximum tumor concentration was reached at 4 h after photofrin solution injection, followed by a decline over the next 8 h. The tumor level of photofrin was significantly higher in the liposomal photofrin group than in the photofrin solution group (1.20 ± 0.35 vs. 0.51 ± 0.17 μg/g; $p < 0.05$). In the skin, the photofrin levels showed a tendency of decrease by liposomalization. Mean tumor/skin ratios were 1.02 in photofrin solution group vs. 3.0 in liposomal photofrin group, respectively. The results demonstrated that the tumor/skin ratio of photofrin levels after liposomal photofrin administration was 3.0 which are higher than that in photofrin solution group. Since the difference between the photofrin concentrations in the tumor and skin is the most crucial issue for the clinical use of PDT, liposomal photofrin can be expected to increase its therapeutic efficacy.

In contrast, the hepatic photofrin concentration was significantly higher in the liposomal photofrin group than in the photofrin solution group ($P < 0.05$). Liposome uptake by the RES in the liver may cause an increase in the hepatic photofrin level. Though adverse histological and biochemical effects on the liver were not shown, the modification of the liposomal composition or PEG modification of the surface of liposomal membrane may enable entrapment in the RES to be avoided without decreasing tumor-specific accumulation.

2.2.2. Antitumor Activity of Liposomal Photofrin

At day 28 after MT-2 implantation into nude mice, MT-2 bearing mice were injected at 10 mg/kg (i.v.) of photofrin or liposomal photofrin, respectively. Laser irradiation was done at 8 h or 24 h after drug injection using a pumped excimer dye laser system (EDL, Hamamatsu Photonics K.K., Hamamatsu, Japan) at 630 nm with a power of 150 mW/cm^2. To generate a high-energy beam with a wavelength of 630 nm, the excimer laser was coupled to a dye laser containing 0.5 mM rhodamine 640 dye in ethanol. Irradiation from EDL was focused using fused silica fibers, which were bent to improve the homogeneity of light distribution throughout the treatment field.

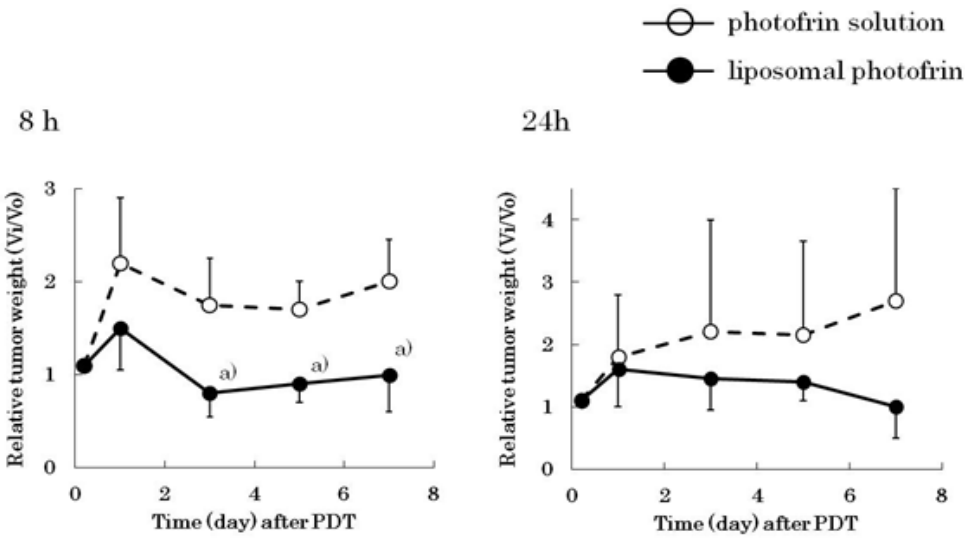

Figure 1. Change of the relative tumor weight after the treatment of PDT. Relative tumor volume (n=4) were calculated at day 1, 3, 5 and 7 after PDT. Laser was irradiated at 8 h or 24 h after injection of photofrin solution (○) or liposomal photofrin (●). The results were expressed as relative mean tumor weight (Vi/Vo), where Vi is the estimated tumor weight at given time and Vo that at the initiation of PDT. Each point represents the mean ± S.D. (n = 4). Significant difference from photofrin solution group is indicated by a) $P<0.05$.

Massive necrosis of tumor tissue was seen at day 3 after PDT with liposomal photofrin, and epithelized at day 7. A significant reduction in the estimated tumor volume was observed in the liposomal photofrin group compared with the photofrin solution group at 8 h after injection, whereas it was not significantly different at 24 h after injection between two group (Figure 1).

The apoptosis index was 6.9 ± 0.4 in the liposomal photofrin group and 2.1 ± 0.4 in the photofrin solution group at 8 h after injection with a significant difference ($P < 0.05$), whereas it was not significantly different 24 h after injection between two groups (3.2 ± 0.5 vs. 2.1 ± 1.2). Endothelial cells in the tumor have a higher oxygen content and/or higher photofrin level and so may be more susceptible to laser irradiation [40]. It is still unclear whether liposomal photofrin accumulates preferentially in vascular endothelial cells as compared with photofrin solution, but the more marked decrease in tumor blood flow after PDT with liposomal photofrin encourages investigation of this possibility. Thus, the hypoxia of tumor cells induced by vascular damage [41, 42] may, at least partly, contribute to the increase in apoptosis. It is well recognized that PDT was reported to induce apoptosis rapidly [43], both in vitro [44, 45] and in vivo [45]. Subcellular damage induced by PDT was reported to affect the cell membrane, cytoplasmic enzymes, mitochondria, lysosomes, microsomes, and nucleus [46-48]. It was reported that photofrin was localized in the mitochondria and not in the plasma membrane in vitro [49]. Photofrin is a ligand for the mitochondrial peripheral benzodiazepine receptor, which is known for its ability to trigger a pore transition [50].

It was also reported that mitochondrial photodamage is followed by an apoptotic response, including changes in the p53 protein level and caspase activation associated with mitochondrial cytochrome c release [51-53]. It suggests that the mitochondria of tumor cells

may be a direct target of photofrin, which may be released from liposomes after internalization by tumor cells. Thus, it is likely that the apoptosis of tumor cells may be induced synergistically by vascular damage followed by hypoxia and mithochondrial injury of tumor cells.

Thus, the liposomalization of the photosensitizer increased its tumor accumulation with a enhancement of the therapeutic effect of PDT for gastric cancer, suggesting that it may offer potential for development of PDT.

2.3. Efficacy of PEG Modified Liposomal Photofrin

To increase the therapeutic index of photofrin, liposomal photofrin (PF-Lip) was modified by 1-monomethoxypolyethyleneglycol-2,3-dimyristoylglycerol (PEG-DMG). Generally, it is considered that a lower trapped ratio is shown in case of liposomalization of the hydrophilic agent by the method of Bangham, since the volume proportion of the inner/outer compartment is very small when it is entrapped in the inner compartment. As photofrin is an acidic drug (pKa = 5.8) and PBS(-) (pH 7.0) are used for hydration, the rate of the ionized form is about 94.1%, so photofrin is easily entrapped in inner compartment of the liposome. However, since the photofrin trapping ratio in both PF-Lip and PEG modified PF-Lip (PF-PEG-Lip) was more than 80%, it was expected that photofrin was entrapped in the liposome membrane, too. Namely, it was suggested that photofrin existed in the liposome membrane and inner compartment.

The survival ratio of M5076 ovarian sarcoma cells after PDT with photofrin solution or each photofrin liposome was evaluated. M5076 ovarian sarcoma cell suspension (1.0×10^5 cells/ml) containing photofrin solution or PF-Lip or PF-PEG-Lip (0-10 μg/ml) was incubated for 1 h at 37 °C. Each sample was exposed to laser light of 630 nm with 2 J/cm^2 by EDL. At every concentration, the photofrin induced cytotoxic effect was photofrin solution < PF-Lip < PF-PEG-Lip, suggesting that photosensitizer induced toxicity was enhanced by liposomalization, especially PEG modification. At the point of 2μg/ml photofrin, survival ratios in PF-Lip (68.6 %) and PF-PEG-Lip (51.4 %) groups significantly decreased ($P < 0.05$ and $P < 0.01$, respectively), compared to that in photofrin solution group (85.7 %).

Then, in order to clarify the enhanced effect of the PEG modification, the uptake of photofrin into tumor cells, ability to generate the singlet oxygen and the release of photofrin from the liposome were examined. On the uptake into M5076 ovarian sarcoma cells, the concentration of intracellular photofrin was the highest in PF-Lip group, whereas that of PF-PEG-Lip was lower than photofrin solution. These results suggested that the layer of PEG existed on the surface of the liposome membrane inhibits the binding of liposomes to tumor cells, and thus prevents internalization of liposomes into the tumor cells.

The photosensitizer loaded into the tumor cells generates singlet oxygen by laser irradiation, and thus necrosis is induced in the cells [14-16]. Namely, it is expected that the affinity of agent to tumor cell, and the ability to generate singlet oxygen is the most important factor of PDT therapy [15].

It is considered that the cytotoxicity of PDT is triggered by the singlet oxygen generated from the photosensitizer [15, 16], and detection of the singlet oxygen is very important in understanding the mechanism of PDT. The amount of singlet oxygen from photofrin solution, PF-Lip and PF-PEG-Lip in 90% CH_2Cl_2 was determined, in comparison to photofrin in

aqueous solvent. On exposure to light of a specific wavelength, the photosensitizer is activated from its ground state S_0 to the excited state S_1. A part of the photosensitizer in the S_1 state transfers to the triplet state T_1 by way of intersystem crossing, and then the photosensitizer in the T_1 state transfers its energy to surrounding triplet oxygen ($^3O_2$), and causes the generation of active singlet oxygen ($^1O_2$). When the singlet oxygen decays to the triplet state, 1270 nm light is released.

Singlet oxygen was detected this emission using a photoncounting method with a high sensitive single channel detector, photomultiplier tube (PMT) or a multichannel detector. Namely, a quartz cuvette filled with each sample was irradiated by laser light (630 nm, 20 mW) generated EDL. Reflected or scattered light from the cuvette was guided to the detection system (Figure 2) which contained a spectroscope, a single channel detector PMT (Hamamatsu Photonics, R5509-42) or a multichannel detector (Hamamatsu Photonics, NIR-PII) and a photon-counter. To separate the 1270 nm emission from the photosensitizer fluorescence, detectors were gated with a delay time from the onset of the laser pulse irradiation and a gate time width, synchronized with laser the pulses. The energy generated at 1260–1280 nm was detected by the system.

The amount of singlet oxygen from photofrin was PF-PEG-Lip = PF-Lip > photofrin solution (Figure 3). It is known that singlet oxygen disappears immediately in aqueous solvents such as PBS(-), and their $T_{1/2}$ is prolonged in hydrophobic solvents such as CH_2Cl_2. Photofrin existed in liposome membrane as hydrophobic environment and the ability to generate singlet oxygen increased. It was suggested that the ability to generate singlet oxygen was influenced in the PDT by liposomalization and the PEG modification.

The photofrin release from each liposome was PF-Lip > PF- PEG-Lip in 50 % FBS/PBS(-) (Figure 4). At the initial 60 min, 79.6 ± 5.4% of photofrin was released from PF-Lip. On the other hand, only 26.0 ± 5.0 % and 60.6 ± 16.3 % of photofrin was released by PF-PEG-Lip at 60 and 180 min, respectively. It was suggested that the release of photofrin was inhibited by PEG-modification.

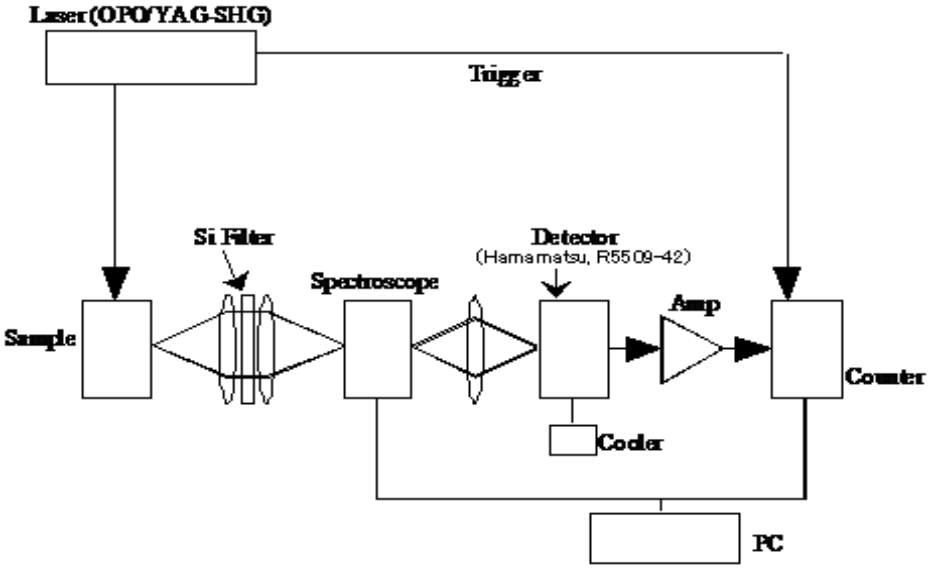

Figure 2. Detection system of 1270 nm emission from the singlet oxygen.

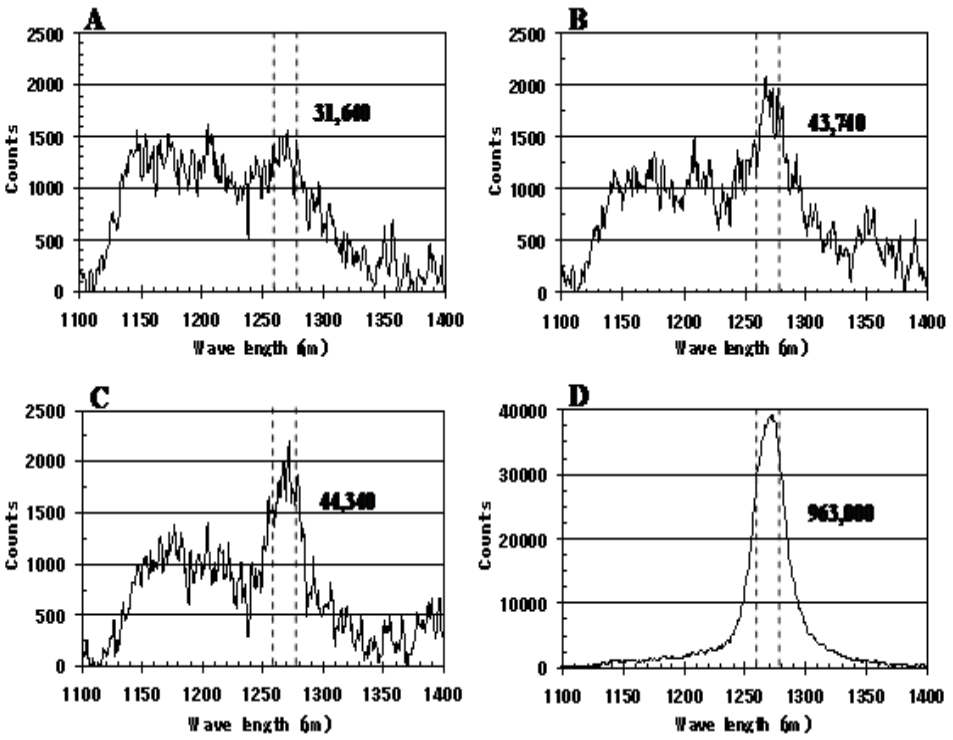

Figure 3. Effect of liposomalization on the generation of singlet oxygen. Each sample was exposed to laser light (630 nm, 20 mW), and the generated singlet oxygen was detected by PMT. Each pattern is expressed as emission of 1270 nm from photofrin. A) Photofrin in PBS B) PF-Lip in PBS, C) PF-PEG-Lip in PBS D) Photofrin in 90% CH_2Cl_2.

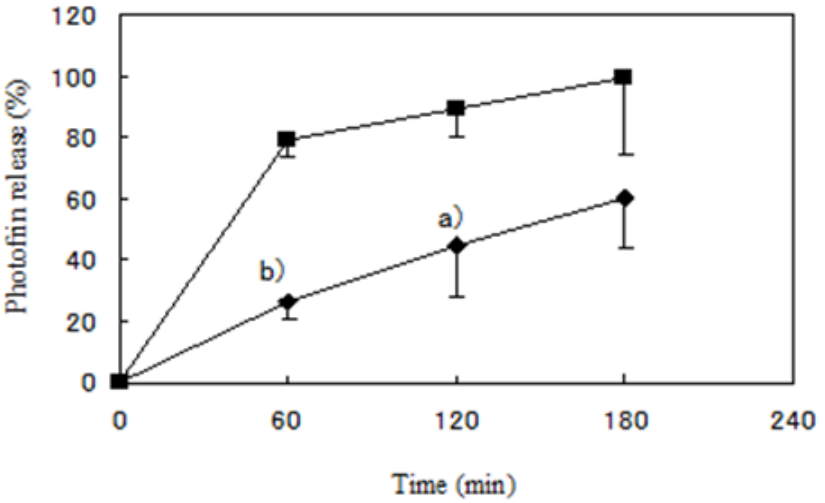

Figure 4. Stability of liposomes containing Photofrin in 50 % FBS. Photofrin release levels are expressed as a percentage of total photofrin in PF-Lip（■） or PF-PEG-Lip（♦. Each sample was incubated at 37°C in 50% FBS. Each point represents the means ± SD (n = 3). Significant differences from PF-Lip are indicated by a) $P<0.01$ and b) $P<0.001$.

In PF-Lip, photofrin was released from the liposome during the incubation period. Since phase transition temperature of DMPC is 23.8°C, PF-Lip may easily release photofrin by incubation at 37°C. These results suggested that photofrin entrapped in the liposome membrane was not stable. Whereas, the PEG modified liposome inhibited the release of photofrin by about half. It was considered that formation of a fixed aqueous layer on the liposome membrane by PEG modification physically changed the stable state of PF-PEG-Lip. Due to this change, PF-PEG-Lip prolonged the photofrin retention time in liposomal membrane. Namely, it was demonstrated that PF-PEG-Lip was effective for PDT in vitro.

It was suggested that liposomalization of agents increases its accumulation in the tumor because of enhanced permeability and retention (EPR) effects [21, 28, 31]. Furthermore, it was known that the PEG modification was effective for the avoidance of RES trapping of liposomes. Therefore, PF-PEG-Lip is expected to produce a higher accumulation in the tumor than PF-Lip, and have a superior PDT effect in vivo, too.

3. Coproporphyrin I

3.1. Liposomalization of Zn-Complexed Coproporphyrin I

In PDT with the photosensitizer and laser irradiation, there are many problems due to the poor solubility of the photosensitizer in water and photosensitivity as an adverse reaction of PDT, although many compounds have been examined as photosensitizer candidates. ZnCPI as a novel photosensitizer was noted. ZnCPI is water soluble and safe. ZnCPI exerts little concentration in the blood of adult humans and was extracted from the meconium in man. As ZnCPI produces active singlet oxygen by laser irradiation, it has been suggested to be an effective tool for PDT [54]. However, ZnCPI is highly water soluble, disappears rapidly from the blood and is difficult to target to the tumor [54]. Furthermore, it was expected that ZnCPI was not easy to use in clinical therapy because of too high sensitivity against pH. It is speculated that liposomalization of ZnCPI is able to solve these problems by the stability of intraliposomal conditions.

The preparation of the ZnCPI liposome was performed according to the Bangham method as a common preparation method. It was expected that the liposome preparations in the different pH of buffers would change the entrapment ratio of ZnCPI, as ZnCPI has four p*K*a's (4.17, 4.43, 4.77 and 4.87). The entrapment ratio of ZnCPI into liposome (PBS(−)) was 10.8±0.3% whereas those in liposomes (lactate buffer) increased, and those in liposomes (pH 4.3, 4.6, 4.82 and 5.0) were 78.0 ± 2.5, 47.2 ± 4.5, 47.1 ± 13.7 and 61.4 ± 1.4%, respectively. However, liposomes were not formed in the lactate buffer (pH 4.0). From these results, the entrapment ratio of ZnCPI into liposomes using lactate buffer (pH 4.3–5.0) was clarified to increase, compared with that using PBS(−).

In general, when a drug is entrapped into a liposome using the Bangham method, the entrapment ratio of hydrophilic drugs is expected to be at a low level, and to be entrapped according to volume ratio between the intraliposomal volume/extraliposomal volume. In the case of using PBS(−), the entrapment ratio of ZnCPI is considered to be at a low level, as ZnCPIwas entrapped into the water phase of the liposome by high ionic form, from the judgement of p*K*a in ZnCPI. On the other hand, the increase of the ZnCPI entrapment ratio

into liposomes (lactate buffer) was thought to be contributed by the high lipophilicity, and then the solubility of ZnCPI into the liposomal membrane. This condition was expected to be suitable for liposomal preparation. However, in the preparation with lactate buffer (pH 4.0), a lipid film was not formed and no liposomal suspension was prepared. It was speculated that this phenomenon was due to the formation of the molecular aggregation of ZnCPI before lipid film formation.

As for the zeta potential of these liposomes, except that in lactate buffer (pH 5.0), the difference of the entrapment ratio of ZnCPI was considered to be caused by the change of the zeta potentials. From these results, the difference of the existing site of ZnCPI into the liposome with the pH of the buffer was discussed. Namely, the change of molecular form - ionic form of ZnCPI occurred due to the change of the pH of the buffer, and the amount of ZnCPI into liposomal membrane changed. The difference of this level was considered to be caused by the change of the zeta potentials. Thus, the ZnCPI liposome using by lactate buffer (pH 4.3) was useful for the pharmaceutical sciences.

The effect of different pH of buffers in liposomal preparation on the ZnCPI distribution in each tissue after each liposome administration was examined. At 2 and 6 h post-injection of the ZnCPI liposome (pH 4.6), the ZnCPI concentration in the plasma of Ehrlich ascites carcinoma bearing mice was shown to be at a high level compared to that in other groups. In contrast, these levels in the liposome (pH 4.3 and 5.0) groups were similar to that in ZnCPI solution group (Figure 5(A)).

In the preparation of ZnCPI liposomes using lactate buffer (pH 4.3), ZnCPI was lipophilic in this condition and was expected to be present in the liposomal membranes. Thus, it is expected that this entrapment site in the liposome induced a high ZnCPI level in the plasma. However, the stability of the ZnCPI liposome (pH 4.3) was not maintained in the plasma. Although this liposome was superior regarding the entrapment ratio in the pharmaceutical properties, it is expected that the drug aggregation was strong due to the high lipophilic properties of ZnCPI at this pH, and the stability of the ZnCPI liposome in the plasma was expected to be lower. In the preparation of the ZnCPI liposome (pH 5.0), ZnCPI had hydrophilic properties and ZnCPI was speculated to exist in intraliposomal space and to be adsorbed onto the extraliposomal surface.

As the adsorbed ZnCPI on the liposomal surface was desorbed in the blood circulation of this liposome, the ZnCPI concentration in the plasma on this liposome group was considered to be low. In vitro experiment, the released level of ZnCPI from ZnCPI liposome in the buffer (pH 5.0) was higher than that in other group. In contrast, pH 4.6 in liposomal condition is the medium value between four p*K*a's of ZnCPI. It is expected that good balance of lipophilicity—hydrophilic as drug carrier was keep in this pH. Namely, ZnCPI liposome (pH 4.6) was shown to be useful in the high entrapment ratio of ZnCPI and the effective blood circulation of ZnCPI.

The ZnCPI concentrations in the tumor after 2 and 6 h of ZnCPI liposome (pH 4.6) treatment were shown to be higher than those in other groups (Figure 5(B)). Namely, it was considered that the ZnCPI liposome (pH 4.6) had the effective antitumor activity by laser irradiation.

Thus, the ZnCPI (pH 4.6) had effective properties of a high entrapment ratio into the liposome, blood circulation and tumor accumulation. Judging from ZnCPI distribution and physical properties, the ZnCPI liposome (pH 4.6) was expected to be useful for PDT. The development of this liposome is expected to contribute to PDT and cancer chemotherapy.

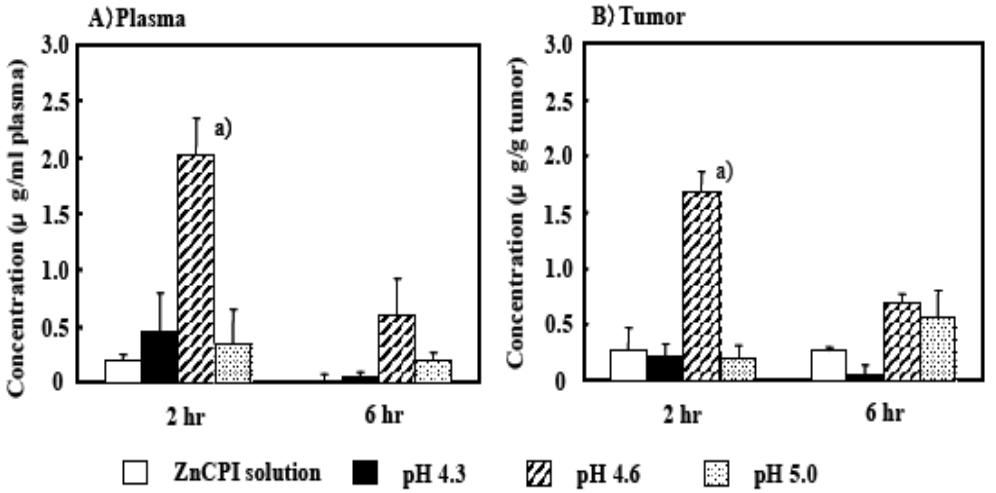

Figure 5. Biodistribution of ZnCPI in tumor bearing mice after liposomal ZnCPI administration in the plasma (A) and tumor (B). Ehrlich ascites carcinoma bearing mice were injected with each liposome (ZnCPI dose : 10 mg/kg). Each column represents the the mean ± SD (n = 3). A significant difference from the level of the ZnCPI solution, pH 4.3 and pH 5.0 group is indicated by a) $P < 0.01$.

3.2. Effect of Liposomalization on the Phototoxicity of Coproporphyrin

The liposomalization of water soluble ZnCPI was attempted and a pharmaceutically useful liposome containing ZnCPI was developed. However, in the liposomalization, it is not necessary to increase the water solubility of drugs entrapped in liposomes. The entrapment ratio was increased by the altered molecular form of ZnCPI in section 3.1. In this section, coproporphyrin I (CPI), which is not suitable for injection due to its hydrophobic properties, was noted. CPI-containing liposomes (Lipo-CPI) and PEG modified Lipo-CPI (PEG-CPI) were prepared, and their efficacies were described.

It was attempted to prepare Lipo-CPI in PBS (-), various buffers with different pH, and lactate buffer. However, in lactate buffer, which was used to prepare Lipo-CPI liposomes, liposomalization of CPI could not be achieved at any pH. As CPI has shown hydrophobic properties at low pH, it is likely that CPI aggregated before formation of the liposomal membrane.

Furthermore, liposomes with a low CPI entrapment ratio were prepared in PBS (-). In contrast, CPI liposomes prepared in 9.0% sucrose/10mM phosphate buffer (pH 7.8) had a high CPI entrapment ratio and were stable. The entrapment ratios of CPI in Lipo-CPI and PEG-CPI were 73.1±9.0% and 91.7±5.6%, respectively, suggesting that it is possible to increase the CPI entrapment ratio by PEG modification. The particle sizes and zeta potentials of Lipo-CPI and PEG-CPI were shown to 142.1 ± 2.1 nm and −38.5 ± 7.7mV, and 142.7 ± 2.4nm and −15.0 ± 2.8 mV, respectively. Namely, in 9.0% sucrose/10mM phosphate buffer (pH 7.8), Lipo-CPI and PEG-CPI could be prepared with a high entrapment ratio of CPI and small particle size.

The tissue distribution of CPI after administration of each liposome was shown in Figure 6. In the plasma, CPI concentrations in the Lipo-CPI and PEG-CPI groups were shown to be higher than in the CPI solution group.

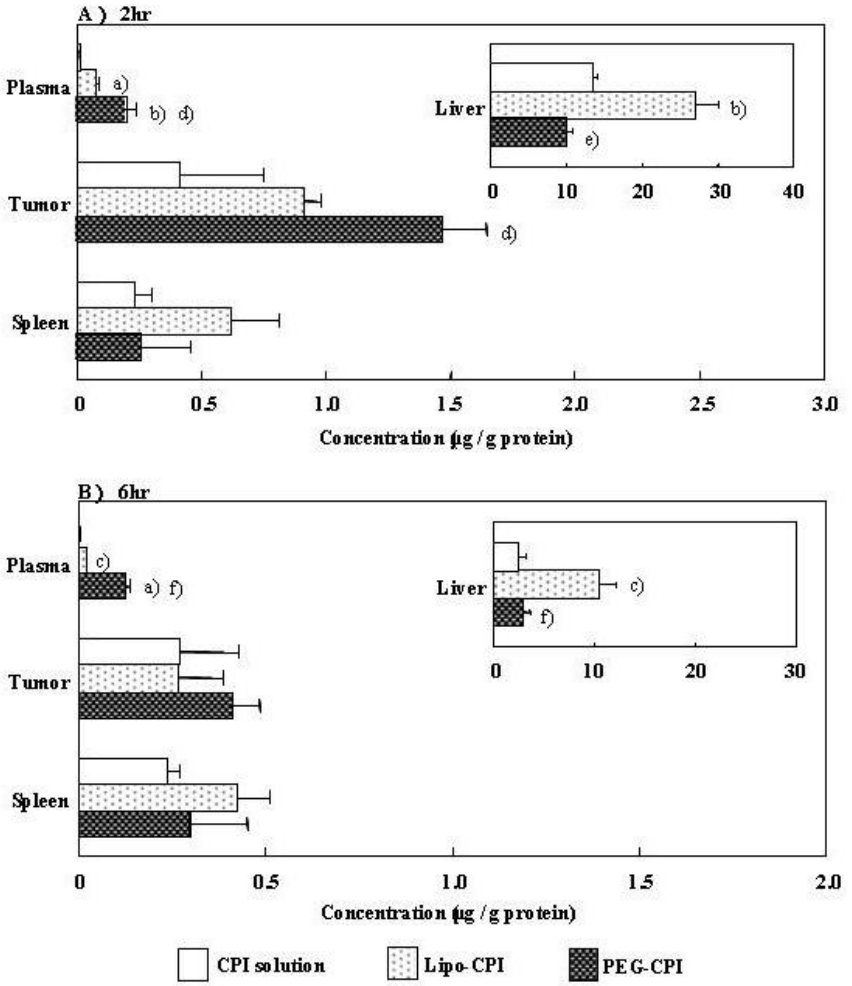

Figure 6. Effects of liposomalization and PEG modification on CPI concentration in plasma and tissues. Ehrlich ascites carcinoma bearing mice were injected with CPI solution, Lipo-CPI, or PEG-CPI (CPI dose: 5.0 mg/kg). Each column represents the mean ± SD (n = 4). Significant differences from the level of the CPI solution group are indicated by a) $P < 0.05$, b) $P < 0.01$ and c) $P < 0.001$, and from the level of the Lipo-CPI group are indicated by d) $P < 0.05$, e) $P < 0.01$ and f) $P < 0.001$.

In particular, CPI concentrations at 2 and 6 h after PEG-CPI injection were 2.5 fold ($P < 0.05$) and 6.5 fold ($P < 0.001$) greater than in the Lipo-CPI group, respectively. Therefore, PEG modification prolonged CPI circulation in the blood. In tumors, the CPI concentration of the PEG-CPI group was higher compared to that in the Lipo-CPI or CPI solution groups. Namely, PEG-modification of liposomes encapsulating CPI probably increased the blood circulation and tumor accumulation of CPI.

In the liver and spleen, the CPI concentrations in the Lipo-CPI group increased compared with those of the CPI solution group, whereas CPI concentrations in the PEG-CPI group were shown to be similar to those in the CPI solution group. Therefore, it is likely that PEG modification of the liposomes formed a fixed aqueous layer around the liposomal membranes, thereby avoiding removal of liposomes by the RES and decreasing the CPI concentration in the liver and spleen.

It is expected that the affinity of photosensitizers to tumor cells and their ability to generate singlet oxygen are the most important factors for PDT therapy [55-59]. PEG modification of liposomes was reported to suppress cellular uptake because of stabilization of the liposomal membranes [60-62]; the cellular uptake of ZnCPI entrapped liposomes was inhibited by PEG modification.

In contrast, the order of the tumor cell uptake of CPI was PEG-CPI > Lipo-CPI > CPI solution. The CPI concentration after 60min incubation with PEG-CPI was significantly higher (1.38 ± 0.13 μg/10^7 cells), compared to that in Lipo-CPI (0.80 ± 0.01 μg/10^7 cells) or CPI solution (0.58 ± 0.06 μg/10^7 cells). Thus, PEG modification of CPI liposomes had a positive effect on tumor cell uptake of CPI. It is speculated that this effect of PEG modification resulted from a change in the interaction with water molecules around the liposomal membrane [63-66].

The order of singlet oxygen generation was PEG-CPI = Lipo-CPI > CPI solution. The liposomalization of CPI probably created hydrophobic conditions that suppressed the water-induced quenching of singlet oxygen, resulting in higher levels of active singlet oxygen.

The order of cytotoxicity by PDT at 60min, which showed a significant difference in intracellular CPI concentration, was PEG-CPI > Lipo-CPI = CPI solution. In particular, the cytotoxicity of PEG-CPI was significantly increased ($P < 0.001$) when compared to that of the CPI solution. Namely, it was considered that this cytotoxicity was reflected the increased CPI concentration in tumor cells with subsequent enhancement of singlet oxygen generation. From these results, it is expected that PEG-CPI had a superior effect on cytotoxicity.

Conclusion

PDT is an effective therapy as a potent and less invasive treatment for early or superficial tumors. However, PDT has the adverse reaction as photosensitivity by accumulation of photosensitizer in the skin. Thus, the improvement of therapeutic index had tried by liposomalization of photosensitizer. Liposomalization and PEG modification of photofrin induced high accumulation of photofrin in the tumor and high generation of singlet oxygen, and was achived superior antitumor effect. Furthermore, PEG-CPI was confirmed to have effective tissue distribution, produce a higher CPI concentration in tumor cells, and enhance the production of singlet oxygen and cytotoxicity by PDT.

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Chapter 7

Antimicrobial Photodynamic Therapy in Periodontics and Implantology

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Abstract

Antimicrobial Photodynamic therapy was first described in the beginning of the 19th century, while studying *paramecium caudatum*, by Raab. Despite this therapy having a great potential for bacterial control, the scientific interest decreased with the description of the antimicrobial effects of penicillin. Since then, the research on photodynamic therapy was polarized on cancer therapy. Recently, with the increased interest on novel approaches on microbial control due to the high incidence of bacterial resistance on usual antimicrobial therapies, the research on Antimicrobial Photodynamic Therapy had gained the attention of various research groups. The use of Antimicrobial Photodynamic Therapy as a local adjunctive therapy on the treatment of periodontal and periimplant diseases has been largely described in the literature in the past 10 years. The objective of this paper is to review the literature on the use of antimicrobial photodynamic therapy on the treatment of periodontal and periimplant disease and to describe the photophysical and photochemical reaction involved on the photodynamic process.

Introduction: Periodontal Disease and Its Challenges

Periodontal Disease is an inflammatory reaction of the tissues surrounding the tooth, usually resulting from gingival inflammation induced by bacteria residing in biofilms on the subgingival tooth surface. Thus, there is formation of a long junction epithelial and connective tissue breakdown due to inflammatory host response and pathogenicity of periodontopathogens, leading to formation of deep sulcus, known as periodontal pockets. The

consequence of this progression is the loss of tooth support due to destruction of Periodontal Ligament and Alveolar Bone, resulting on tooth loss. Although subgingival biofilm and specific bacteria are needed to induce attachment loss, periodontal disease is considered a multifactorial disease, suffering influence of the host response and being modified by genetics, systemic conditions such as diabetes mellitus and environmental conditions (smoking habits, social condition).

When referring to Periodontal Disease and its multiple factors, we should not consider it as a classical infection, but as an opportunist. The evidence point that certain virulent bacterial species are responsible for most of periodontal destruction. They are grouped on the red and orange complexes (Socransky et al., 1998).

Periodontal inflammation may provoke tooth loss and consequent life quality decrease. Besides, there are evidence about systemic consequences of periodontal breakdown, raising the attention to periodontal disease as a public health problem and an increased interest on novel treatment strategies to achieve better treatment outcome.

Periodontal therapy is, actually, divided two mean aspects: removal of biofilm deposits and host response modulation. The standard therapy, since we know periodontology as a dental specialty, consists in plaque/calculus removal from root surface by the scaling and root planning procedures.

Scaling and root planning (SRP) can be performed with manual curettes and ultrasonic devices. It is the first step on the causal related periodontal treatment and is also called Non-surgical treatment. When reevaluating the success of the therapy, after non-surgical therapy, if there is still bleeding on probing of the periodontal pockets, a surgical approach is indicated with the objective of proper removal of the remaining subgengival calculus and plaque and a healing without residual deep pockets. Although appear a simple procedure, scaling and root planning becomes difficult when deep periodontal pocket are to be instrumented, specially due to anatomical complexities such as furcation areas and root sulcus, that are physical barriers for a good root instrumentation, increasing the possibilities of residual calculus and needs of a surgical approach (Adriaens and Adriaens, 2004). Thus, innumerous efforts are made to detect and remove residual calculus (Krause et al., 2003).

In an attempt to overcome the inefficiency of SRP, the use of systemic and local antibiotic was proposed (Slots and Rams, 1990). Systemic antibiotics has been indicated for several situations involving periodontal therapy such as diabetic patients (O'Connel et al., 2008), Chronic Periodontitis (Silva et al., 2011; Sampaio et al., 2011), and Aggressive Periodontitis (Mestnik et al., 2011). The adjunctive use of local antibiotics has also been used in challenging sites (Blands et al., 2010). A serious concern on the use of systemic antibiotics in periodontics is the development of bacterial resistance, a common and serious problem nowadays (Greentein, 2005). When used systemically, antibiotics can act on the needed periodontal site, but it acts on other oral and body sites, which is not desirable. In addition there is a possibility of adverse effects and interaction with other medication. The advantage of locally applied antibiotics is the site specificity, the mean disadvantage is the cost and the possibility of bacterial resistance is not totally excluded. In addition, the effective use of antibiotics demands an antibiogram, since we're treating a polymicrobial disease. Thus, it becomes more difficult to choose a correct antibiotic regimen, considering that, although the general periodontal infection are due to certain bacterial specimens, there is variability of antibiotic resistance in different strains. For this reason a broad spectrum antimicrobial therapy seems to be useful when treating periodontal disease. This chapter will present a

novel antimicrobial local therapy for treating periodontal and periimplant disease, Antimicrobial Photodynamic Therapy, its basic principles and a proposed treatment protocol.

Antimicrobial Photodynamic Therapy: Basic Principles

Historic Background

Photodynamic Therapy (PDT) was firstly described by Raab almost 100 years ago (Raab, 1900). When studying a culture of *paramecium caldatum* in na acridine dye, Raab noted that the light exposure of the culture lead to a lethal photosensitization. Thus, he postulated that the energy transfers from the light to the dye, as the process that occurs on plants with chlorophyl. Despite the good potential of this technique, its clinical practice was suppressed by the penicillin description by Alexander Fleming. Thus, the lethal potential of PDT was concentrated on cancer diagnosis and therapy, and several dyes and light sources were tested and developed. An attempt to destroy tumor cells was made using hematoporphyrin and X ray radiation as an electromagnetic radiation source. Although success was not achieved, this experience has demonstrated the need of resonance from a light source and the photosensitizer. After that, several attempts were made with polychromatic and conventional lamps as a light source, but the inconvenience of the thermic component and energetic inefficiency was reported. Recently, the use of PDT as a antimicrobial approach has gained the attention of the scientific community (Wainwright, 1998) as an alternative to common antibiotic treatment. In this chapter, we will refer to this approach as Antimicrobial Photodynamic Therapy (aPDT).

The Photodynamic Mechanism

The mechanism behind aPDT involves photochemistry. The photodynamic process is based on the activation of molecular oxygen to generate a reactive oxygen (Singlet Oxygen, hydroxyl radical, superoxide anion, and hydrogen peroxide), all of them with cytotoxic effects. The energetic transfer is made by a photosensitizer.

Photosensitization is a light mediated process that requires a dye resonant with the light source (photosensitizer), initiating a series of processes in an naturally nonabsorbent substrate, in aPDT, bacterial cells. Thus, the first step is the photon absorption by the dye on its original ground state, promoting it to a singlet, more energetic and reactive, state. From this phase, the photoprocess can gain two pathways, the decay of the dye to its ground state leading to a phenomenon known as fluorescence, or, a system intercrossing that leads to the promotion of an even more energetic state, also called triplet state. The triplet state is more stable than the singlet because of a spin conversion of the more external electron. This characteristic allows the dye on its triplet state sufficient time to perform chemical reactions, leading to lethal photosensitization.

When occurring, the photodynamic process has two main pathways type I and II. Type I derives from the electron or hydrogen transfer between the dye triplet state and other

molecules. This process produces reactive molecules (superoxides, hidroxyperoxiles, and hidroxil radicals such as hydrogen peroxyde) that are lethal to the target cell; after that, the dye returns to its ground state. The Type II photoprocess occurs with a spin change between the triplet dye state and the diogyxene triplet state. This reaction produces cytotoxic excited singlet oxygen, meanwhile the oxygen returns to its ground state. Both processes cause biomolecule oxidation, but the singlet oxygen is the main photodynamic therapy mediator.

Light Sources in aPDT

The most common light source used in aPDT is LASER (Light Amplification by Stimulated Emission of Radiation). It is important to explain how a laser light is produced and what justify it use. As commented before, laser is an acronym and it is an electromagnetic energy that has special proprieties. The production of laser is based on the Stimulated Emission Theory, postulated by Albert Einstein in 1917. In accordance to Einstein, an excited state of the atom, when colliding to a photon, emits instantly an identical photon following two basic rules: the photon has the same direction of the stimulator photon; the wave of both, stimulated and stimulator are synchronized and the wavelengths are equal, its magnitudes are summed and raise the light intensity. This process produces a monochromatic, coherent and high intensity light. Due to its monochromacity, laser light has been preferred as a light source to be used on aPDT, because, if the laser light is resonant with the dyes absorption peak, an optimal efficiency could be achieved.

Three lasers with different wavelength, HeNe (630nm) and AsGaAl (665 and 830 nm) were tested with a phenothiazine dye 0.01% w/l. The results pointed to a high efficiency with 665nm, proving the resonance importance to optimize aPDT results (Chan and Lai, 2003).

Although the laser light has been preferred as a light source, we cannot exclude the possibility of using another monochromatic light sources such as Light Emmiting Diode, that is monochromatic and, in addition, a cheaper and economic technology.

In an attempt to use alternative light sources for aPDT, some authors have compared a Xenon red filtered lamp with a HeNe Laser). Despite the equivalence of the treatments, the xenon lamp needed 10% more light delivery to achieve the same results, what increase the local temperature (Matevski et al., 2003).

Photosensitizers

As mentioned before, the aPDT requires the activation of a photosensitizer by a proper wavelength light source. The human tissues can transmit efficiently light in the red spectral band (between 630 and 700nm) resulting in a deeper penetration. Thus, most of the photosensitizers used in aPDT are resonant with a red light source as shown in table 1.

An ideal photosensitizer should have affinity for microbial cells, a broad spectrum action to act efficiently in polymicrobial infections, minimizing the possibilities of inducing resistant strains or to promote mutagenic processes. Besides that, it should be possible to indentify a therapeutic window that: supresses extensively the pathogenic microorganism with minimal damage to host cells and prevent pathogen proliferation after treatment (Konopka and Golinski, 2007).

Table 1. Phenotiazinium photosensitizer and their respective maximum absorption wavelength

Photosensitizer	λ_{max}
Methylene Blue	*656 nm*
Dimetil Methylene Blue	*648 nm*
Toluidine Blue O	*625 nm*
Malaquite Green	*654 nm*

Most of the dyes used on aPDT are phenotiazines, with aromatic tricyclic rings on its structures and are cationic. Most of them exihibt a significant singlet oxygen release and inflicts microbial photodamage in acceptable concentrations for topical and intravenous use.

Neutral and anionic dyes can effectively inactivate gram positive bacteria, but, when treating gram negative microorganism they link with the external membrane, but cannot inactivate when light activates. Thus, the positive charge, characteristic of cationic molecules, seems to be important for photoinactivation.

The positive charge a planarity of phenotizines allows them to link with DNA, what in the past, lead us to believe that the phototoxicity was only due to DNA damage. Today, it is accepted that in both, gram negative and positive, the bacterial membrane is the target structure.

It is important to highlight that the photosensitizer light absorbance characteristics should not coincide with endogenous cromophores. Light on 400-500 nm wavelength absorbance peaks should not be used without an optic fiber because of the lack of penetration in deep sites mainly because this spectral band is well absorbed by hemoglobin. In addition, wavelength greater than 900 nm are well absorbed by water, restricting the therapeutic window to a 600 to 900 nm band.

In a comparative *in vitro* study, the production of singlet oxygen, lypophilicity and bactericidal capacity were tested in several dyes: Methylene Blue, Toluidine Blue, Proflavine, Dimetil-Methylene Blue, Acrydin Orange, Malaquite Green (MB, TBO, PF, DMMB, AO, MG). It was reported that, in decrescent order, DMMB, MB, TBO, and MG were capable of producing singlet oxygen, AO was not capable of producing detectable amounts of singlet oxygen. The bactericidal effect did not totally follow the effectiveness of producing singlet oxygen. Although DMMB has a greater production of singlet oxygen, MB had greater bactericidal effect. In terms of lipophilicity, TBO and MB showed hydrophilic characteristics, meanwhile AO and DMMB showed lipophilic characteristics (Sayed et al., 2005).

Although innumerous dyes are available, this chapter will focus on the use of phenotiazinium dyes, which are naturally cationic molecules and are available for commercial use as products for aPDT.

Phenotiazinium Dyes

The phenotiazines are blue dyes, such as Methylene Blue and Toluidine Blue O. The size and structure of phenotiazines, when compared to other aromatic compounds, make them excellent binders to DNA. The photodynamic action caused by MB in DNA seems to be selective to guanosine residues (Wainwright, 1998).

Although they´ve shown excellent binding to DNA, MB and TBO only bind to simple species, like bacteriophages. Against bacteria, the dye structure seems to influence on bacterial photodynamic site of action.

The cellular absorption can be determined by a combination of charge, distribution and lipophilicity, these characteristics can be controlled on the dye synthesis, changing the photochemistry behind the dye action and its proprierties of photobleaching, photodynamic efficacy, oxyreduction potential, hidrophylic/lipophilic balance, planarity and charge.

Physical and Chemical Parameters

Photosensitization Efficacy

The capacity to erradicate bacterial cells depends on the ability to generate cytotoxic effects. The quantum liberation is the parameter that measures this ability. This characteristic can be modified and optimized by a change on the molecule structure. An example is the methylation of the molecule.

Another strategy is called Heavy Atom Effect, which improves the efficiency by the stabilization of the cromophore triplet form when a component of the molecule is substituted by an element of higher atomic weight. However, we need to know that molecule modification can affect not only the quantum liberation but also the molecule structure, factor that can change the action site as well, or determine the dye's non-absorption by the target cell.

Photobleaching

Photobleaching, also called photodegradation, is the degradation process of the cromophore by the light. The exposure of MB and TBO to light can cause photodegradation and, as consequence, the formation of leuco molecules (reduced dye molecules), that are not resonant to the light source, reducing the photodynamic efficacy.

Hydrophilic / Lypophilic Balance

The hidrophylic character is determined by a logarithmic mathematical formula (Figure 1). Compounds like MB and TBO have LogP < 0, characterizing a hydrophilic compound. Species with LogP > 1.5 are considered lipophilic and compounds with LogP between 0 and 1.5 are considered amphiphilic.

$$\mathrm{Log}\,P = \mathrm{Log}\left\{\frac{\left(A - A^{1}\right)}{A^{1}} \times \frac{V_{\mathrm{w}}}{V_{\mathrm{o}}}\right\}$$

Figure 1. *Log*P. A and A^1 are intensity of absorption before and after of the partition; V_w and V_o are the volume of the respectives on water and 1-octanol. LogP measure the hydrophilic/lipophilic balance of a compound.

Planarity

Planarity is affected by the spacial distribution of the atom on the molecule, affecting its three dimensional aspect. A change on molecule radicals to alter the photodynamic efficacy or another characteristic such as lipophilicity should be done with attention, because the

addition or exclusion of a radical can change the molecule spacially, changing its binding site and cellular entrance.

Microbiological Parameters

Bacteria are grouped in two mean groups in accordance to response to Gram staining process, being gram positive (G+) or gram negative (G-). These bacterial groups show structural differences between them.

Gram negative bacteria show greater resistance to aggressive agents such as antibiotics. Although they have thinner cellular walls, it is more complex than gram positive bacteria.

The effectivity of aPDT is dependent of the bacterial group. Gram positive bacteria are more susceptible to photodynamic inactivation than Gram negative (O'Neill, 2002; Meisel and Kocher, 2005). The outer membrane seems to have great importance in this resistance difference. It has high permeability to hydrophilic molecules. Lipopolysaccharides and porin channels seem to work as a selectivity barrier.

The difference between two bacterial groups can be explained by the mechanism involved on cromophore uptake by target cells, which is described in two pathways: pathway number 1 is known as a direct translocation of the photosensitizer through the plasmatic membrane and it is present when the target cells are gram positive bacteria and tropozoic stage protozoa; pathway number 2 requires an initial augmentation of the cellular wall permeability and occurs on gram negative bacteria, yeast and cystic stage protozoa. In all these cases the main binding force between the photosensitizer and wall surface negative charge functional groups has an electrostatic nature and the process is completed in a substantially short time of preirradiation (Wainwright, 1998; Jori et al., 2004).

Although the differences between bacterial groups, we need to highlight that, when dealing with Periodontal Disease, we are confronting a polymicrobial infection with no planktonic bacteria, but a well formed dental biofilm.

Dental biofilm has a intermicrobial matrix with complex diffusion channels where occurs nutrient permutation between colonies, in addition to nutrition cooperation, these channels make antibiotic diffusion difficult as well as its biding to target cells.

Factors Influencing Antimicrobial Photodynamic Therapy

Bacterial cells have 4 development phases during it life cicle. A initial phase called LAG, with a low growing rate, corresponds to an adaptation period. Then LOG phase, with an exponential growing rate, followed by stationary phase, characterized by a nutritional limitation and equality between replication and death rates. The last phase is characterized by logarithmic fall on bacterial count, when death rates are greater then replication. Some authors attribute differences on photodynamic action for each physiological phase (Bhatti et al., 1997).

The effects of different physiological phases and ambient (saline, saliva and bovine serum) were tested *in vitro* with gram negative bacteria and TBO as the photosensitizer. The presence of bovine serum reduces the photodynamic effectivity, low pH had deleterious effect

on treatment and no difference was reported regarding the bacterial physiological phase (Kömerik and Wilson, 2002).

The authors explained differences with a possible deleterious effect of protein components to aPDT due to protein absorption of light, reducing cromophore light exposure and consequent reduction of cytotoxic radical production. In addition, protein can compete with bacteria to dye molecule binding.

An *in vitro* study tested TBO in cultures of *Porphyromonas gingivalis*, comparing the photodynamic action on pH variation, pre-irradiation time, light dose and physiological stage. A dose dependent effect on light expousuring was reported, a higher dye concentration did not raise photodynamic killing, probably by cellular saturation and light absorption in non-target sites (Bhatti et al., 1997). When compared to saline, the presence of equine serum on the culture reduced 5 fold the photodynamic action.

A possible negative effect of high concentration can be explained by a common characteristic of phenothiazine dye called metachromasy (appearance of bands of absorption due to aggregation on aqueous solution) (Bergeron, 1958).

Metachromasy occurs because of an aggregation tendency of phenothiazine, in general there is involvement of van der Walls forces, intermolecular hydrogen linkage, hydrogen-solvent linkage, hydrophobic interactions and dispersion forces, becoming difficult to highlight an specific force. These aggregated complexes have a different absorption band than the monomer, reducing the photodynamic efficacy as they do not absorb on the wavelength used on aPDT.

The presence of bacteria seems to decrease the monomers absorption spectrum by, like dye concentration, altering the monomer/dimer proportion. This alteration causes a shift on absorption spectrum. The hypochromic effect seems to be proportional to bacterial concentration (Usacheva et al., 2003).

Strategies of Optimization

It was previously showed that innumerous factors can affect aPDT outcome. A number of strategies has been proposed to improve treatment results. Often, those strategies involve molecular modifications, as cited on *Physical and Chemical Parameters* section, a pretreatment of the target site or antibody attachment on dye molecule.

An attempt to improve photodynamic damage was made adding a photomechanical wave while irradiating. The result showed a 2 fold increased penetrance on oral biofilm *in vitro*, after 10 photomechanical pulses and a 99% of bacterial death (Soukos et al., 2003). The explanation to this phenomenon could be an deformation on biofilm microcolonies and alteration of the micro channels, improving dye penetration and photodynamic action on target sites.

Another strategy proposed is the conjugation of long chain molecule to the dye structure (Soukos et al., 1998). Conjugation of a 37 lysin chain to a neutral dye results to photoinactivation of both gram negative and positive bacteria. The use of short chain molecules (8 peptides) did not perform the same effect (Hamblin et al., 2002).

The use of EDTA as a pretreatment has potential to improve photodynamic action. It causes an ionic repositioning of Mg^{2+} and Ca^{2+}, neutralizing negative charges and promoting electrostatic repulsion, disorganizing cellular membrane. Adoption of EDTA as a

pretreatment can improve dye penetration on bacterial cell (Soukos et al., 2003; Jori et al., 2006).

Linkage of dye-target was improve by conjugation of monoclonal antibodies for *P. gingivalis* and TBO. The test was performed *in vitro* on presence of fibroblasts and *S. sanguis*, achieving substancial and preferential killing of periodontopathogens (Bhatti et al., 2000).

Preclinical Studies

In this section the results of preclinical studies will be presented. Most of them are the basis for the rationale use of Antimicrobial Photodynamic Therapy, thus an important step to understanding the science behind this novel treatment.

In Vitro Studies

The importance of the charge on photodynamic efficacy was tested when using $chlorin_{e6}$ conjugates with positive, neutral and negative charges. As experimental target, were used human epithelial cells, *P. gingivalis* and *A. viscosus*. Regarding the lethal sensitization with positive, neutral and negative charged conjugates, *P. gingivalis* had reductions of 99%, 91% and 78%, respectively. The cationic (positive) conjugate achieved 99.99% reduction of *A. viscosus* without affecting human epithelial cells. This paper proved therapy security, since the photosensitizer had presented a 20 to 100 fold predilection for bacterial cells when compared to human cells (Soukos et al., 1998).

The effect of aPDT on bacterial proteases of *P. gingivalis* on culture of peripheral mononuclear cells (PMN) was tested. The authors reported a dose dependent decrease of bioactivity of lipopolysaccharides accompanied by a reduction on the production capacity of IL-8 from PMN. The photosensitizer used was TBO and it was suggested that it has a possible biological activity on LPS (Kömerik et al., 2000).

The results were confirmed in another paper that presented a dose dependente reduction of proteolytic (Arg-X) activity. With a high dose (111.4J/cm²) and a total energy of 126J, no proteolytic activity was detected. The irradiation with a total energy of 21J resulted in 99.9% of killing. The irradiation with TBO in a concentration of 6.25µg/ml caused decrease of Arg-X activity, with a light dose of 167.1J/cm² and total energy of 180J, its activity was reduced by 88% (Packer et al., 2000).

A study evaluated the effect of aPDT on formed biofilm by Confocal Scanning Laser Microscopy (CSLM). A bacterial reduction of 97.4% when TBO 25 µg/ml and 31.5J total energy was observed. The author reported a greater lethal photosensitization on biofilm external layers, attributing it to a difficulty of the cromophore to penetrate and diffuse through internal layers (O'Neill et al., 2002). The importance of resonance between cromophore and light source was tested *in vitro*.

The light sources used were HeNe laser (632.8 nm) with an output power of 30mW was used, a AsGaAl diode laser with 100mW (665 and 830 nm) with multiple irradiation times. The results showed that, with the irradiation of HeNe laser for 30 seconds (3.2J cm²), was

possible to eliminate 55 to 81% of the total amount of bacteria. When the exposure timewas aumengted to 60 seconds (6.4 J/cm²) it was possible to increase killing rates (79 to 81%). The infrared Laser Diode showed the worst results, with the highest exposure time, it was only possible to reduce 40 to 55%. The most effective light source was red Laser Diode (665 nm) with a killing rate of 71 to 88% with 30 seconds exposure time (10.6 J/cm²) and 95 to 100% when a 60 seconds (21.2J/cm²) exposure was used. This data confirms the importance of resonance on aPDT performance (Chai and Lai, 2003). Despite the exposure time was the same for diode laser and HeNe, for a better scientific study design, the output power should be equal, for an example, with 30 seconds, the diode laser delivered a light dose larger than the HeNe in 60 seconds. Besides that, it was noted that the infrared band could not achieve desirable killing rates because of the lack of resonance with the photosensitizer..

Some authors had tested the importance of power density (53, 106, 159 and 212 mW/cm²), dye concentration (0.01, 0.1, 0.5, 1, 2.5 and 5 mg/ml) and energy density (3, 6, 12 and 24 J/cm²) on aPDT performance. The best treatment performance was achieved with the combination of a 159 mW/cm² power density with 1 mg/ml dye concentration and a light dose of 12 J/cm² (Qin et al., 2008). The killing rate became higher as the dye concentration was raised from 0.01 to 1 mg/ml, but no improvement was noted with concentrations higher than 1mg/ml, probably because of the dimerization.

In Vivo Studies

A controlled *in vivo* study in rodent model was performed to secure the safety of aPDT. The test group received the treatment with TBO 1mg/ml, preirradiation time of 5 minutes in accordance to a previous study from the same research group (Qin et al., 2008), with a higher light dose (159mw/cm² and energy density of 60J/cm²) in order to achieve optimal antimicrobial results; control group 1 received a high laser dose (140 J/cm²); control group 2 received high concentration of TBO (2.5 mg/ml) without irradiation; and control group 3 received no treatment as a negative control. The results reported that the treatment produced no harmful results on periodontal tissues, dentin or pulp (Luan et al., 2009). These results are in accordance with a previous in vitro study that involved co culture of S. *sanguis,* keratinocytes and fibroblasts (Soukos et al., 1996).

Another study in a rodent model tested efficacy and biodistribution of aPDT. TBO as a photosensitizer at three concentrations (0.01, 0.1 and 1 mg/ml) with a HeNe laser irradiation, and a total energy of 48 J was used. The authors reported no damage on the periodontal tissues and, as expected, as the total energy delivered was raised, the killing rate became more effective. In addition, it was noted that TBO tissue penetration is concentration dependent, and the epithelial keratinized layer seems to act like a barrier, concentrating TBO and avoiding deeper penetration on adjacent connective tissue (Kömerik et al., 2003). The photosensitizer's ability to penetrate epithelium is desirable since is known that periodontopathogens penetrates this tissue (Socransky and Haffajee, 2002).

A histologic and radiographic study in rodent model was performed using MB at 0.1mg/ml, red diode laser (685 nm), preirradiation time (PIT= 1 minute). The results showed statistical differences at 5 and 15 days, but no difference was found at 30 days. The ligature was not removed during all experimental times (Almeida et al., 2007).

Almeida et al. (2008) made a histomorphometric evaluation of bone loss in a rodent model furcation defect with the same parameters of a previous study (Almeida et al., 2007) and an energy density of 4.5J/cm². Animals were divided in 4 groups, 1 test (aPDT) and 3 controls (no treatment, laser treatment and photosensitizer) with 3 experimental times each group (7, 15 and 30 days). At the first experimental time, aPDT showed better results, but in the last experimental time all groups showed the same bone loss. Again, the ligature was not removed, a fact that can explain the lack of difference on late experimental times.

A study in rats was designed to compare aPDT with scaling and root planning (SRP). It was used TBO 1mg/ml with a red diode laser (635 nm) and an energy density of 12J/cm². The positive control group received SRP and negative control group received no treatment. The results showed no differences between treatment groups (Qin et al., 2008). Despite that, it is important to highlight that aPDT received no mechanical treatment, preserving biofilm integrity, what may cause, as we've seen on a previous section, lack of effectivity of aPDT, if we extrapolate to a clinical situation like a furcation involvement that sometimes the mechanical instrumentation cannot cleanefficiently, a liquid photosensitizer could be desirable to achieve microbial reduction on this areas, acting like an adjuvant therapy.

Another study in rodent model compared aPDT as an adjuvant to SRP and SRP with no adjuvant treatment in diabetic (D) and non-diabetic (ND) mice. Test group received aPDT (irrigation with TBO 0.1mg/ml and irradiation 24J energy (4J/site) with a red diode laser), con. Sacrifices were made at 5, 15 and 30 days to perform histologic and histomorphometric analysis. In both groups the aPDT subgroup showed less bone loss in all experimental period, when a intergroup comparison was made, ND mice treated with SRP presented more bone loss than D from aPDT group (Almeida et al., 2008).

The aPDT was tested in immunosuppressed mice as an adjunct to SRP. Animals were divided in 2 groups (Normal and Immunosuppressed) and 3 subgroups (SRP+TBO, SRP and SRP+aPDT). The protocol for aPDT was (0.1mg/ml TBO, PIT of 1 minute, 4J per site in a total of 24J of energy in a red diode laser 635nm).

Histological analysis has shown, in SRP Normal group (N), a disorganized connective tissue with a high number of degenerated neutrophil, thin bone trabecule and reabsorption areas. On the other hand, SRP immunosuppressed group (I) presented disorganized connective tissue with small number of fibroblasts with an intense inflammatory infiltrate and cement reabsorption. When histological analysis was made at both aPDT subgroups presented an intact periodontal ligament with parallel collagen fibers and a few inflammatory infiltrate. Bone tissue showed thin and organized trabecule without reabsorption signals as well as cement surface. On intergroup comparison SRP subgroup of normal mice showed more bone loss than aPDT immunosuppressed mice (Fernandes et al., 2009).

A microbiological and immunological study in dogs was conducted in order to compare aPDT, SRP and SRP+aPDT. Thus, microbiological samples were collected at baseline, 1, 3 and 4 weeks after treatment, as well as gingival biopsies for immunological analysis. There was no difference between groups regarding cytokine response. On microbiological analysis showed an intensive reduction on the first week on SRP+aPDT group but not in later periods. All groups showed no difference on bacterial load reductions, but groups treated with aPDT showed an in intense reduction of *Aggregatibacter actinomycetemcomitans* (Oliveira et al., 2011).

Considering that *a.a.* has the ability to penetrate epithelium, this finding can be explained by photosensitizer's capacity of penetration and action trough pocket epithelium (Kömerik et al., 2003). Some authors had raised the killing specificity of aPDT in *a. action-mycetemcomitans* by adding a targeting moiety and achieved, on biofilms, targeted killing (Suci et al., 2010).

Some authors had designed preclinical animal studies to evaluate the capacity of aPDT to treat periimplantitis. It is important to highlight that periimplant inflammatory disease is caused by the same pathogens responsible by periodontal tissue loss (Mombelli and Décaillet, 2011). Although microbiologically similar, periimplantitis seems to differ from periodontitis by a singular characteristic of continuous periimplant tissue destruction (Martins et al., 2005; Albouy et al., 2009).

A preliminary study in dogs tested the ability of aPDT to perform microbial reduction in experimental periimplantitis. The protocol consisted in scaling implant surface followed by treatment with TBO 0.01% and irradiation with a 685nm diode laser, power of 50mW and a total energy of 4J. The results showed considerable microbial reductions and, in some samples, total elimination of bacteria (Shibli et al., 2003). These results were accompanied by a successful re-osseointegration when associated with Guided Bone Regeneration (GBR) procedures using expanded polytetrafluoroethylene (ePTFE) (Shibli et al., 2003b).

The use of aPDT as a single compared with a standard treatment of periimplantitis was published in 2005. On this study in beagle dogs, the conventional treatment used as control group was Implant scaling followed by chlorhexidine 0,12% irrigation, after raising a total flap. The aPDT group received surgical access, followed by treatment with 0.01% w/w azulene paste (25% w/v azulene solution in a base paste composed of 10% urea peroxide, 15% detergent, and 75% carbowax vehicle) with a red diode laser (660nm), 40mW of power, and a total energy of 7.2J for 3 minutes, without mechanical treatment. Microbiological results showed no difference between treatments (Hayek et al., 2005).

Another comparative study was performed in order to investigate the re-osseointegration rates between standard treatment and adjuvant aPDT on experimental periimplantitis lesions in association with GBR procedures.

The control group received mechanical treatment after raising a total flap; the test group received the same treatment and aPDT treatment with TBO 0.01%, diode laser 830nm wavelength, 50mW power output and 80 second of irradiation performing 4J/cm² of energy density. The aPDT group achieved a better re-osseointegration rate than control group. Another interesting finding was that there were less membrane exposures on test group (Shibli et al., 2006). These results may be due to aPDT ability to disinfect treated implants surface, since a biostimulatory effect on osteoblast is not expected when using phenothiazine chlorides and laser (Stein et al., 2009).

Clinical Studies

There is number of clinical publication regarding the use of aPDT in periodontics. In this section it will be discussed the treatment protocols proposed on literature, and the results found.

Chronic Periodontitis

The first clinical publication was a split mouth design study comparing SRP, aPDT, SRP+aPDT and Oral Higiene Instructions. Ten patients with one tooth with 4mm pocket depth per quadrant were recruited. The study reported no randomization and the treatment procedures were: SRP, oral hygiene instruction and aPDT with or without SRP. The aPDT parameters were: one minute mouth rinsing with MB 0.005% five times (0, 2, 4, 9 and 11 days) with a red diode laser 685nm, 30mW output power, for 1.11 minute and 1.6J/cm² energy density. Clinical and microbiological analysis were performed showing significant reduction only on SRP groups, with no difference between them. The clinical results were followed by microbiological results (Yilmaz et al., 2002).

These results may be explained by the photosensitizer application by mouth rinsing, leading to a photosensitizing of only supragingival plaque and, as cited before the presence of protein on saliva could compete with MB for microbial binding sites (Bhatti et al., 1997; Kömerik et al., 2002). Another factor was that 4 mm pockets in unirradicular teeth aren't challenging as furcation on mutirradicular teeth or deep pockets on unirradicular teeth (more than 4 mm). These facts could explain the lack of difference between SRP and aPDT adjunctive to SRP.

A randomized controlled clinical trial tested adjunctive aPDT treatment of periodontal maintenance patients with ≥ 4 mm periodontal pocket. Thus, 24 maintenance patients were divided in to groups (SRP or SRP+aPDT). The aPDT group received a protocol with diode red laser 670nm, power density of 75mW/cm², and an comercial phenotizine chloride 10mg/ml, with 1 minute PIT, followed by irrigation with saline solution and 60 seconds of irradiation time per tooth (10 seconds each periodontal site). The results showed significant reduction of *F. nucleatum* and *E. nodatum* at three month analysis and *t. denticola* at six month analysis. There were no relevant differences between changes in pocket depth and clinical attachment levels. Despite, bleeding on probing showed greater reductions on aPDT group (Chondros et al., 2008).

On the other hand, a similar randomized controlled clinical trial tested the treatment outcome of a single episode of aPDT on subjects diagnosed with chronic periodontitis. Clinical and microbiological outcomes were evaluated showing differences favouring test group on bleeding on probing and no significant differences on microbiological parameter (Christodoulides et al., 2008). It is important to highlight that absence of bleeding on probing on consecutive examinations is considered an important outcome regarding periodontal inflammation.

A split mouth design study compared SRP with or without aPDT adjunctive treatment. There were compared clinical outcome and Gingival Crevicular Fluid at 0, 1 week and 3 months period. These study showed better results than previous reports with the same aPDT protocol (Chondros et al., 2008; Christodoulides et al., 2008) with significant differences favouring test group on all clinical parameters, not only bleeding on probing (Braun et al., 2008). The same photosensitizer of previous studies was used, but the laser power output was 100mW, PIT 3 minutes and 10 seconds of laser irradiation per site.

A randomized clinical trial on parallel group tested the adjuvant effect of aPDT after ultrasonic SRP. The aPDT protocol included irrigation with a commercial phenothiazine chloride 10mg/ml, a PIT of 3 minutes, followed by irrigation with saline solution and irradiation with 680nm red diode laser, 75mW output power for 1 minute each site with

vertical movements. The energy density was not described on this paper. For inclusion criteria, subject must have at least 4 sites positive for presence of *P. gingivalis* and probing pocket depth of 4 to 8 mm. There were no differences regarding clinical or microbiological outcome between treatments, bleeding on probing did not achieved statistical significant difference, despite there was a numerical difference in favor to test group (Polansky et al., 2009).

On the other hand, a double blind placebo controlled randomized clinical trial on periodontal maintenance patients with residual pocket depth ≥ 5mm. Both groups received manual SRP; control group received photosensitizer application and irradiation with non-resonant laser light; test group received dye application with a commercial phenothiazine chloride with a PIT of 3 minutes, followed by irrigation with distilled water and irradiation with a 680nm red diode laser with 75mW/cm² power density for one minute each site. The procedure was repeated in 1, 2, 7 and 14 days after initial treatment. Clinical evaluations were made at 1, 3, 6 and 12 months. Plaque index did not shown intergroup difference indicating same plaque control in both groups. Test group exhibited statistical difference on clinical outcomes (Probing Pocket Depth, Clinical Attachment Level and Bleeding on Probing) raising the variable of number of applications of aPDT efficacy (Lulic et al., 2009).

The short-term effect of aPDT on diabetic uncontrolled patient was compared with two different therapies. This study involved 45 diabetic patients with moderate to severe periodontal disease and uncontrolled type 2 diabetes and there were evaluated clinical outcomes e HbA1c levels. Subjects were randomly assigned in three treatment groups group 1 received SRP, group 2 received SRP associated with doxycycline 100mg in a period of 14 days in accordance to a previous publication (O'Connell et al., 2008), group 3 received SRP+ aPDT, MB 0.01%, diode red laser 670nm, no pre irradiation time was described on paper, the pocket was illuminated for 60 seconds, the laser power output was not described on the paper. The results showed no difference regarding treatment outcome on group 1 and 3. Only SRP + doxycycline (group 2) showed significant difference between baseline and 3 months regarding HbA1c (Al-Zahrani et al., 2009).

Antimicrobial Photodynamic therapy showed superior results on short-term reduction of *F. nucleatum* during full mouth disinfection procedure in patient with localized chronic periodontitis, improving clinical and microbiological parameters significantly during 3 months follow up. A phenothiazine chloride dye was used and a red diode laser with 660nm wavelength and 60mW/cm² power density and optical fiber, PIT was 60 seconds and irradiation time was 10 seconds per site with 60 seconds per tooth, for full mouth disinfection the tongue was treated as a tooth, with irradiation at 6 segments for 10 seconds each (Sigusch et al., 2010).

A study aiming to evaluate the capacity of aPDT to reduce bacterial viability in periodontal pockets using an aqueous solution of 50% toluidine blue at 0.005 mg/500 ml and 50% Endo PTC injected within the periodontal pocket with a 3 minutes PIT, irradiation was applied with a 632.8 nm low-intensity red diode laser at 4 J/cm² with an optical fiber, for 60 seconds in the interior of the periodontal pocket, with vertical movements. Samples were collected before and after SRP, and after aPDT. The results showed statistical difference favoring aPDT, with 81.24% to SRP and 95.90% after aPDT application (Pinheiro et al., 2010).

A randomized clinical study with a parallel design was proposed aiming to evaluate clinical effects of aPDT for SRP within 3 months follow up. Thus 58 subjects with moderate chronic periodontitis were randomly assigned in three groups: A, received SRP; B, received SRP + aPDT in a single application (immediately after SRP); and C received SRP + aPDT with two applications (immediately after SRP and 6 weeks after treatment). The aPDT protocol included insertion of 0.01% methylene blue as photosensitizer into the instrumented periodontal pockets, followed by irradiation with a 670-nm wavelength and 140-mW output power diode laser equipped with single-use light-diffusing tip for 60 s. With these settings, the dose of the laser is about 6 J and the energy density is about 21 J/cm². The results showed no difference between groups at 3 months evaluation regarding all clinical parameters. There was statistical difference on Bleeding on Probing ate te3 month evaluation in favor of aPDT groups, but there was no intergroup difference on aPDT groups (Ge et al., 2011).

The results found in this study are in accordance of previously published clinical trials (Chondros et al., 2008; Christodoulides et al., 2008; Braun et al., 2008), but the multiple application did not showed relevant difference as a previous multiple application publication (Lulic et al., 2009). This difference could be explained by several reasons such as photosensitizer concentration and number of applications or light dose. Although, treating the same pathological entity, the published studies are not clear regarding laser application and dosimetric parameters used, becoming difficult a comparison between them.

A split mouth design clinical trial tested the effect of aPDT and irrigation with photosensitizer as adjunctive to SRP. A total of 33 subjects were recruited and three non-contiguous tooth with moderate to severe periodontal loss. The selected teeth were randomly assigned in three groups: SRP, SRP+TBO, SRP+aPDT. Clinical parameters and micro-biological samples were collected at 60, 90 and 180 days. Results regarding microbiological aspects, aPDT group showed significant reduction of positive sites for periodontopathogens, when clinical parameters were compered, there was no significant intergroup difference (Theodoro et al., 2011).

Aggressive Periodontitis

Aggressive periodontitis is a rapidly progressing disease that affects otherwise healthy individuals (Armitage and Cullinan, 2010), is characterized by episodic and rapid bone loss and inadequate host response to periodontopathogens, inducing a high susceptibility to periodontal disease. Use of adjunctive antibiotic therapy is commonly associated to a higher success of aggressive periodontitis treatment (Feres et al., 2001).

Antimicrobial photodynamic therapy was firstly propose as a novel therapeutic strategy to treat aggressive periodontitis in a pilot split mouth study design as a single therapy, being compared to SRP. On this randomized controlled clinical trial, 10 subjects diagnosed with generalized aggressive periodontitis were treated with SRP or a single application of aPDT (phenothiazine chloride 10mg/ml, 660nm of wavelength red diode laser at 60mW/cm² power output, combined with an optical fiber, with 1 minute PIT followed by irrigation with distilled water and 10 seconds irradiation per site). The clinical parameters were measured at baseline and 3 months after treatment and inter and intragroup results were compared (Oliveira et al., 2007).

Both therapies resulted in improvement of clinical parameters at 3 month evaluation. Intergroup comparison had failed to show significant difference between therapies. A clinical relevant data was the time consumption in both therapies, the average time spent on SRP group was 8 minutes per tooth, meanwhile the average time on aPDT group was 3 minutes. Another interesting data was the ability of aPDT as a single therapy, despite its noninvasiveness, achieve similar results to SRP and sometimes, numerical advantage although no statistical relevance was found.

A study with the same design aiming to investigate cytokine levels in the gingival crevicular fluid (GCF) at 7 days before and 0, 1, 7, 30 and 90 days after treatment was performed and TNF-α and RANKL were measured in ELISA immunoassay (Oliveira et al., 2009).

The same aPDT protocol of a previous study was applied (Oliveira et al., 2007). The results presented were in accordance with previously described clinical results. The TNF-α level showed a time related decrease levels after treatment. The RANKL levels showed a non-significant decrease over time. The intergroup comparison failed to show significant difference between treatments although there were numerical differences in favor of aPDT on 1, 30 and 90 days post treatment.

Continuing the analysis of the clinical and immunological evaluation, a microbiological profile was performed to compare both treatments. Microbiological samples were collected at 7 days before, at baseline, and 90 days after treatment. The results showed an increase in the mean counts between 7 days before, and baseline as between baseline and 90 days. Despite there was no statistical difference between total microbial counts between treatments, it appears that both treatment showed selectivity to different bacterial species. Meanwhile aPDT showed marked reductions on *a. actinomycetemcomitans* and recolonization with *T. forsythia* and *P. gingivalis* was noted, on SRP group there were significant reductions on *red complex* bacteria, but the levels of *A.a.* remained high. These results leaded to the suggestion of a combination of the two therapies on treatment of Aggressive periodontitis on a larger study (Novaes Jr. et al., 2011).

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Chapter 8

Cell Death and Resistance Mechanisms Triggered by Photodynamic Therapy

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Abstract

Photodynamic Therapy (PDT) was the first drug-device combination approved by the US Food and Drug Administration (FDA) almost 2 decades ago, but even so remains underutilized clinically. In principle, PDT is a simple adaptation of chemotherapy that consists of 3 essential components: photosensitizer (PS), light, and oxygen. None of these is individually noxious, but together they initiate a photochemical reaction that culminates in significant toxicity leading to cell death. Antitumor effects of PDT derive from 3 inter-related mechanisms: direct cytotoxic effects on tumor cells, damage to the tumor vasculature, and induction of a strong inflammatory reaction that can lead to the development of systemic immunity (Agostinis et al, 2004).

PDT is frequently regarded as a dual specificity treatment. The selectivity is achieved by an increased PS accumulation within the tumor as compared to normal tissues and by the fact that illumination is limited to a specified location. Several possible mechanisms of selective PS retention within tumors include greater proliferative rates of neoplastic cells, a lack of or poor lymphatic drainage, high expression of LDL receptors on tumor cells (many photosensitizers bind to LDL), low pH (which facilitates cellular uptake), increased vascular permeability, or tumor infiltration by macrophages that are efficient traps for hydrophobic photosensitizers (Moan and Berg, 1992; Moan and Peng, 2003). Therefore, selectivity is derived from both the ability of useful PSs to localize in neoplastic lesions and the precise delivery of light to the treated sites (Figure 1). A

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limitation of PDT is that it cannot be a curative procedure for large and disseminated tumors. Nonetheless, even for an advanced disease it can improve the quality of patients' life and prolong survival. In addition, PDT can be performed repetitively without the accumulation of serious side effects, and it can be combined with most other treatment modalities. Its minimally invasive nature and scar free wound-healing have made it a good option for the treatment of skin cancers and other skin disorders (Szeimies et al, 1996b; Szeimies and Landthaler, 2002).

Finally, many PDT procedures can be performed in an outpatient or ambulatory setting, thereby not only alleviating costs, but also making the treatment patient-friendly. The only adverse effects of PDT relate to pain during some treatment protocols and a persistent skin photosensitization that has been circumvented by the newer agents (Agostinis et al, 2011).

This chapter will address the most important biological aspects of PDT, photophysics and photochemistry, different modes of cell death, resistance to therapy and impact of PDT on angiogenesis as the growing tumor´s favorite

The Principles of Photodynamic Therapy

Photodynamic Therapy (PDT) was the first drug-device combination approved by the US Food and Drug Administration (FDA) almost 2 decades ago, but even so remains underutilized clinically. In principle, PDT is a simple adaptation of chemotherapy that consists of 3 essential components: photosensitizer (PS), light, and oxygen. None of these is individually noxious, but together they initiate a photochemical reaction that culminates in significant toxicity leading to cell death. Antitumor effects of PDT derive from 3 inter-related mechanisms: direct cytotoxic effects on tumor cells, damage to the tumor vasculature, and induction of a strong inflammatory reaction that can lead to the development of systemic immunity (Agostinis et al, 2004).

PDT is frequently regarded as a dual specificity treatment. The selectivity is achieved by an increased PS accumulation within the tumor as compared to normal tissues and by the fact that illumination is limited to a specified location. Several possible mechanisms of selective PS retention within tumors include greater proliferative rates of neoplastic cells, a lack of or poor lymphatic drainage, high expression of LDL receptors on tumor cells (many photosensitizers bind to LDL), low pH (which facilitates cellular uptake), increased vascular permeability, or tumor infiltration by macrophages that are efficient traps for hydrophobic photosensitizers (Moan and Berg, 1992; Moan and Peng, 2003). Therefore, selectivity is derived from both the ability of useful PSs to localize in neoplastic lesions and the precise delivery of light to the treated sites (Figure 1). A limitation of PDT is that it cannot be a curative procedure for large and disseminated tumors. Nonetheless, even for an advanced disease it can improve the quality of patients' life and prolong survival. In addition, PDT can be performed repetitively without the accumulation of serious side effects, and it can be combined with most other treatment modalities. Its minimally invasive nature and scar free wound-healing have made it a good option for the treatment of skin cancers and other skin disorders (Szeimies et al, 1996b; Szeimies and Landthaler, 2002).

Finally, many PDT procedures can be performed in an outpatient or ambulatory setting, thereby not only alleviating costs, but also making the treatment patient-friendly. The only

adverse effects of PDT relate to pain during some treatment protocols and a persistent skin photosensitization that has been circumvented by the newer agents (Agostinis et al, 2011).

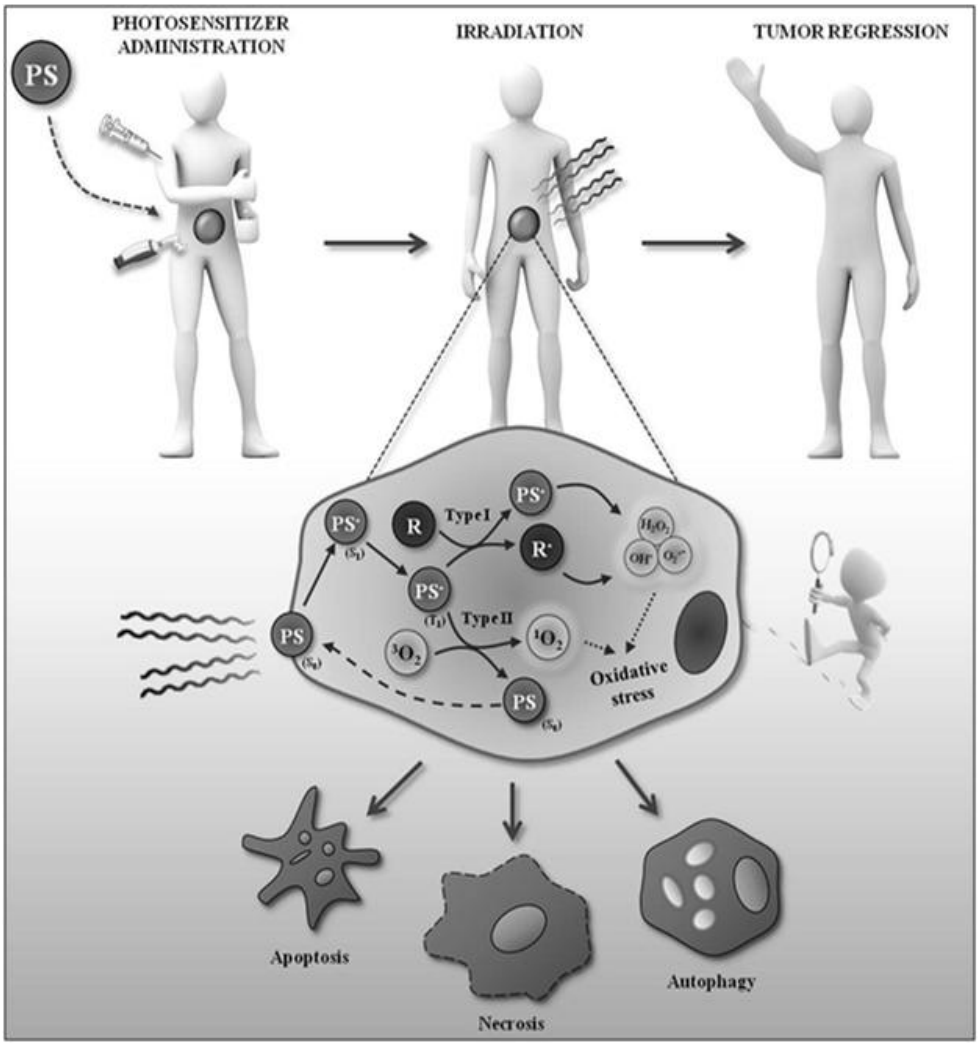

Figure 1. Photodynamic Therapy of Cancer: Mechanism of action. The PS, which is not harmful to the body in its original state, is administered to the patient, by topical or systemic application, and accumulates in malignant tissues. Subsequently, the abnormal tissue containing the PS is exposed to specific wavelengths of light. The PS in its singlet ground state (S_0) becomes activated to an excited singlet state (S_1), which is followed by intersystem crossing to an excited triplet state (T_1). The excited PS (PS*) can undergo two kinds of reaction. First, it can react directly either with the substrate (R), such as the cell membrane or a molecule, transferring a hydrogen atom to form radicals (R*). The radicals interact with oxygen to produce ROS ($O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$) (type I reaction). Alternatively, the PS* can transfer its energy directly to oxygen, to form singlet oxygen ($^1O_2$), a highly ROS. These species oxidize various substrates (type II reaction). These ROS mediate oxidative stress which cause extensive cellular damage. Thus, photosensitized cells died, mainly by necrosis, apoptosis or autophagy, leading to tumor regression. H_2O_2: hydrogen peroxide; $O_2^{\cdot-}$: superoxide ion; $^1O_2$: singlet oxygen; $OH^{\cdot}$: hydroxile radical; PS: photosensitizer; PS*: excited photosensitizer; R: organic substrate; R*: radical; ROS: reactive oxygen species; S_0: singlet ground state; S_1: excited singlet state; T_1: excited triplet state.

This chapter will address the most important biological aspects of PDT, photophysics and photochemistry, different modes of cell death, resistance to therapy and impact of PDT on angiogenesis as the growing tumor´s favorite.

Photophysycal and Photochemistry Processes in PDT

The absorption of light energy by a PS increases the energy of its electrons rendering the PS excited. The excited PS can relax back to its ground state by emitting fluorescence, or to a triplet state through a process called intersystem crossing, from which it can relax by emitting phosphorescence.

This phenomenon is currently being exploited for the development of photodiagnostic procedures (Stringer and Moghissi, 2004). In the triplet state, the excited PS can transfer its energy to molecular oxygen, one of the rare compounds which have triplet ground state, and the two molecules relax to respective singlet states. In the singlet state molecular oxygen, $^1O_2$, is excited, highly reactive and thereby responsible for the majority of lesions generated during PDT (Sharman et al, 2000). Excited PS on transferring its excess energy returns to its ground state to accept further photons or becomes photochemically degraded (used) in a process referred to as photobleaching.

Alternatively, an excited photosensitizer may react directly with biomolecules to form free radicals that further react with molecular oxygen producing superoxide radical anion (O_2•-), hydrogen peroxide (H_2O_2) or hydroxyl radical (HO•). Superoxide can react with the hydroxyl radical (HO•) to form singlet oxygen ($^1O_2$), or with nitric oxide (NO^-) (also a radical) to produce peroxynitrite ($OONO^-$), another highly reactive oxidizing molecule (Castano et al, 2004). These reactive oxygen species (ROS), together with singlet oxygen produced pathway, are oxidizing agents that can directly react with many biological molecules (Figure 1).

Basically two types of mechanisms can occur after photoactivation of the PS. One involves the generation of free radicals (type I photochemical reaction) and the other involves the production of $^1O_2$ (type II) which is the main photoproduct responsible for the cell inactivation. The type II reaction has an important effect on cell death, although the photodynamic mechanism of the sensitizer on neoplastic tissues is not fully understood. These radicals from type I may further react with oxygen to produce reactive oxygen species. Both Type I and Type II reactions can occur simultaneously, and the ratio between these processes depends on the type of PS used, the concentrations of substrate and oxygen (Bilski et al, 1993; Ma and Jiang, 2001; Castano et al, 2004).

Tumor Photosensitizers: Still an Ongoing Research Issue

Most of the PSs used in cancer therapy are based on a tetrapyrrole structure, similar to that of the protoporphyrin contained in hemoglobin. The ideal photosensitizer should meet

several criteria: chemical purity, preferential tumor retention, fast tumor accumulation and rapid clearance, activation by light with good tissue penetration, high absorption coefficient, no dark toxicity, minimal or absent skin photosensitivity, low manufacturing costs and good stability in storage. No photosensitizer with such characteristics has been found but these criteria delineate the area for development. The first PS to be clinically employed for cancer therapy was a water-soluble mixture of porphyrins called hematoporphyrin derivative (HPD), a purified form of which, porfimer sodium, later became known as Photofrin. Although porfimer sodium is still the most widely employed PS, the product has some disadvantages, including a long-lasting skin photosensitivity and a relatively low absorbance at 630 nm (Agostinis et al, 2011). These factors have stimulated research leading to the development of second generation photosensitizers (Alvarez et al, 2006; Yslas et al, 2005, 2007), and at the present time there is such a great number of potential PS for PDT.

Protoprophyrin IX (PpIX) is a natural photosensitizer that can be made by the human body. It is possible to induce the biochemical production of photosensitizing amounts of this natural compound in diseased tissues by the introduction of a small and simple amino acid, namely 5-aminolevulinic acid (ALA). Although ALA has no intrinsic photosensitising properties, it is metabolised to produce PpIX (the active agent in ALA–PDT). The metabolic conversion of exogenous ALA into a photosensitizing concentration of PpIX is relatively fast, requiring only 1–3 h. This is the primary reason for the tissue specificity found with ALA-PDT. PpIX is synthesized by the mitochondria, the primary source of energy for the cell. Oxidative damage to such structures interferes with energy metabolism and can lead to cell death.

ALA can be administered as ALA hydrochloride or as ester derivatives. The ester derivatives have different polarities and some derivatives can be useful to treat deeper lesions, since some esters can penetrate deeper into the lesion. The methyl-ester derivative is approved in Europe and Australia for treatment of skin disorders (actinic keratosis and basal cell carcinoma, Metvix® by Photocure, Norway and Galderma, France), the hexyl-ester derivative is approved in Europe for endoscopic fluorescence diagnosis of bladder cancer (Hexvix®, Photocure). Aminolevulinic acid and its methylester, methyl aminolevulinate (MAL), are second-generation photosensitisers (Kennedy, 2006; Krammer et al, 2006; Alvarez et al, 2007; Fayter et al, 2010).

There has been a major effort among medicinal chemists to discover second-generation PSs, and several hundred compounds have been proposed as potentially useful for anticancer PDT. The majority of PS used both clinically and experimentally, is derived from the tetrapyrrole aromatic nucleus found in many naturally occurring pigments such as heme chlorophyll and bacteriochlorophyll. Naturally occurring porphyrins are fully conjugated (non-reduced) tetrapyrroles and vary in the number and type of side groups particularly carboxylic acid groups (uroporphyrin, coproporphyrin and protoporphyrin). There are a set of classical chemical derivatives generally obtained from naturally occurring porphyrins and chlorins that include such structures as purpurins, pheophorbides, pyropheophorbides, pheophytins and phorbins some of which have been studied (a few extensively) as PS for PDT.

A second widely studied structural group of PS is the phthalocyanines (PC), and to a lesser extent, their related cousins the naphthalocyanines. Several PC have been evaluated by our group on both in vitro (Yslas et al, 2007; Rumie Vittar et al, 2008) and in vivo (Rumie Vittar et al, 2008; Yslas et al, 2009) tumor models.

Another broad class of potential PS includes completely synthetic, non-naturally-occurring, conjugated pyrrolic ring systems. These comprise such structures as texaphyrins (Detty et al, 2004), porphycenes (Szeimies et al, 1996a), and sapphyrins (Kral et al, 2002). A last class of compounds that have been studied as PS are synthetic dyes or non-tetrapyrrole derived naturally occurring. Examples of the first group are toluidine blue O (Stockert et al, 1996) and Rose Bengal (Bottiroli et al, 1997; Chan, 2011; Panzarini et al, 2011) and from the second group are hypericin (from St Johns wort) (Agostinis et al, 2002).

Natural Photosensitizers

Natural constituent's purificated from plants have always been of great importance in traditional medicine. Many drugs used for cancer treatment have been isolated from natural products (Gordaliza, 2007), using plants as the main source. In this context, several compounds isolated from plant can be mentioned: vincristine, irinotecan, etoposide and paclitaxel, while actinomycin D, mitomycin C, bleomycin, doxorubicin and l-asparaginase are drugs obtained from microbial sources, and citarabine, trabectedin-ET-743, bryostatin-1, neovastat from marine sources (Nobili et al, 2009).

Active vegetal compounds are first evaluated on in vitro models, and then tested for their efficacy through in vivo studies. The most important strategies for the selection of plant in cancer drug discovery include random screening, ethnomedical knowledge and chemo-taxonomic information (Lautie et al, 2008).

Paclitaxel and docetaxel are clinically well-stablished natural chemotherapies used in the treatment of several solid tumours (ovarian, breast, lung, head and neck, gastroesophageal, bladder, testis, endometrium neoplasms). Both vegetal drugs are active administrated as single agents or in combination chemotherapy (Mekhail & Markman, 2002; Ramaswamy & Puhalla, 2006). Others naturals drugs, such as vincristine, vinblastine and vindesine, have been largest used in the treatment of lymphatic tumors, as well as in some solid tumours (Nobili et al, 2009). Aditionally, it have been demonstrated that curcumin and reveratrol are potential chemopreventive and chemotherapeutic agents (Duvoix et al, 2005; Johnson & Mukhtar, 2007). Regarding to anthracyclines, doxorubicin exhibits interesting anti-tumor activity and is widely used in the treatment of breast cancer, small cell lung cancer, ovarian cancer and lymphomas. Daunorubicin and idarubicin are mainly used for the treatment of leukaemias, while they show activity also in lymphomas or breast cancer (Robert, 2005).

The application of natural constituents against cancer has increased therapeutic efficacy. In recent years it has been shown that many bioactive phytochemicals are photosensitizers; their toxic actions against organisms such as viruses, micro-organisms, insects or cells are increased by light of certain wavelengths. During the last century, the photodynamic effects of certain natural occurring compounds, such as alkaloids and phenolic, have been described (Nobili et al, 2009).

Hydroxyquinone pigments isolated from plant present principal attention as photosensitizers for photodynamic therapy. The photosensitizer activity of hypericin was firstly recognized due to cutaneous photosensitivity in animals following ingestion of high quantities of hypericum plants and subsequently exposure to sunlight (Giese, 1980). The diverse photodynamic action of hypericin targets a range of carcinoma cells (Vandenbogaerde

et al, 1997, 1998; Blank et al, 2000; Ali et al, 2001; Ali and Olivo, 2002; Höpfner et al, 2003; Kamuhabwa et al, 2004; Cole et al., 2008).

Our group has evaluated other natural compounds, such as anthraquinones isolated of the vegetal specie *Heterophyllaea pustulata*, that exhibited photosensitization reactions on mice Balb/C when were administrated orally and irradiated with sunlight (Núñez Montoya et al, 2008). In addition, these anthraquinonic derivatives showed photodynamic activity on human breast cancer cells in vitro (Comini et al, 2011). The photosensitizer hypocrellin-A obtained from *Hypocrella bambusae*, and aloe-emodin, a similar hypericin structure, presented phototoxicity on lung carcinoma cell line (Chio-Srichan et al, 2010; Lee et al, 2010).

PDT Oxidative Damage Triggers Different Modes of Cell Death

Precisely why cells die when subjected to the PDT-generated ROS has been the subject of intense investigations in recent years. Different cell types, different PS and different incubation and illumination conditions can all significantly alter the outcome of PDT. The question of whether there are particular cellular proteins that are more susceptible to oxidation by singlet oxygen or other PDT-generated ROS is just starting to be addressed. The precise way that PDT influences these cellular death pathways, is largely governed by where in the cell the PS is located because ROS have a short half-life and act close to their site of generation. This subcellular localization in turn is governed by the chemical nature of the PS (molecular weight, lipophilicity, amphiphilicity, ionic charge and protein binding characteristics), the concentration of the PS, the incubation time, the serum concentration and the phenotype of the target cell (Castano et al, 2004). Upon exposure of cancer cells to the photodynamic stress, multiple signaling cascades are concomitantly activated and depending on the subcellular location of the generated ROS and the intensity of the oxidative damage, they dictate whether cells will cope with the stress and survive or succumb and die.

PDT can evoke the 3 main cell death pathways: apoptotic, necrotic, and autophagy-associated cell death (Figure 1). Apoptosis is a generally major cell death modality in cells responding to PDT (Oleinick et al, 2002; Dolmans et al, 2003; Buytaert et al, 2007; Agostinis et al, 2011). Based on this idea, we have reported evidence about PDT apoptosis occurrence (Alvarez et al, 2005, 2006; Rumie Vittar et al, 2008). Agarwal et al. were the first to report apoptosis after PDT with chloroaluminum phthalocyanine in mouse lymphoma L5178Y cells, and found a rapid induction of apoptosis mediated by phospholipase C activation (Agarwal et al, 1991). This type of death, identified in single cells usually surrounded by healthy-looking neighbors, and characterized by cell shrinkage, blebbing of the plasma membrane, the organelles and plasma membrane retain their integrity for quite a long period. In vitro, apoptotic cells are ultimately fragmented into multiple membrane-enclosed spherical vesicles. In vivo, these apoptotic bodies are scavenged by phagocytes, inflammation is prevented, and cells die in ‘immunological control’ of necrosis resides within or outside cells. Apoptosis, requires transcriptional activation of specific genes, include the activation of endonucleases, consequent DNA degradation into oligonucleosomal fragments, and activation of caspases (Castano et al, 2005). PDT induces apoptosis via two major pathways: mitochondria-mediated or intrinsic pathway, and death receptor-mediated or extrinsic pathway (Almeida et

al, 2004). The mitochondrial apoptosis pathway occurs mainly when photosensitizers localizing in these organelles are used. Nonetheless, the intrinsic pathway may also be activated when other cell structures are direct targets of photodynamic action (Almeida et al., 2004).

The first stages of the pathway are disruption of mitochondrial transmembrane potential and release of cytochrome c to the cytosol. This allows formation of a complex called apoptosome and activation of hydrolytic enzymes (caspases). The pathway is strongly influenced by the Bcl-2 family of proteins (Oleinick et al, 2002). Activation of these hydrolytic enzymes leads to the cleavage of multiple cellular proteins, DNA fragmentation and eventually cell death (Almeida et al, 2004). Death receptor-mediated apoptosis is considered to occur preferentially when photosensitizers targeting the cell membrane are used. It is triggered by multimerization of cell membrane receptors belonging to the tumor necrosis factor (TNF) receptor superfamily. Of all the members of the family, Fas receptor is thought to play a major role in PDT induced apoptosis (Oleinick et al, 2002). Phototoxicity is not propagated only through caspase signaling but involves other proteases, such as calpains, as well as nonapoptotic pathways (Buytaert et al, 2007). Regarding calpains, we have shown that a new water-soluble phthalocyanine derivative triggers an alternative mechanism of caspase-independent apoptosis in photosensitized cells (Rumie Vittar et al, 2010).

Necrosis has been referred to as accidental cell death, caused by physical or chemical damage and has generally been considered an unprogrammed process. It is characterized as a violent and quick form of degeneration affecting extensive cell populations, characterized by cytoplasm swelling, destruction of organelles and disruption of the plasma membrane, leading to the release of intracellular contents and inflammation. In necrosis, decomposition is principally mediated by proteolytic activity, but the precise identities of proteases and their substrates are poorly known (Castano et al, 2005). Factors that promote necrosis include extra-mitochondrial localization of PS, high dose of PDT, and glucose starvation (Almeida et al, 2004; Dellinger, 1996; Kiesslich et al, 2005; Oberdanner et al, 2002). In response to therapy, we have noted that doubling light dose triggering apoptosis lead to necrotic cells death in porphyrin photosensitized cancer cells (Alvarez et al, 2005).

Since these first descriptions of necrosis and apoptosis, it has become evident that the situation is somewhat more complicated with alternative modes of cell death being described. These include mitotic cell death (Castedo et al, 2004), cells exhibit multiple aberrations including retardation at G (2)-M, increased cell volume, and multinucleation; programmed necrosis (Bizik et al, 2004; Vanlangenakker et al, 2008), the cysteine cathepsin-(cathepsins B or L) mediated lysosomal death pathway (Berg and Moan, 1994; Leist et al, 2001; Reiners et al, 2002), and autophagic cell death (Yu et al, 2004; Buytaert et al, 2006; Kessel and Oleinick et al, 2009; Reiners et al, 2010), in which a normal function used to degrade components of the cytoplasm is involved and which is characterized by autophagosomes, autolysosomes, electron-dense membranous autophagic vacuoles, myelin whorls, multivesicular bodies, as well as engulfment of entire organelles. These last cell death mechanisms have been recent described with PDT (autophagy and programmed necrosis).

On the other hand, autophagy is defined as a process of programed cell survival that consist of degradation of cellular components in double membrane structures called autophagosomes, this undergo a maturation process that culminates in fusion to lysosomes to form autolysosomes, where cytoplasmatic components are degraded and recycled which provide essential building blocks, such as amino acids back to cell (Kang et al, 2011).

Autophagy optimizes nutrient utilization in rapidly growing cells when faced with hypoxic or metabolic stresses and, hence it contributes to normal and cancer cell survival (Jin & White, 2008).

This event is known to display a dual contrasting function in cancer cell biology because it may be decreased in tumor cells compared with normal cells. Initial studies appreciated autophagy as a tumor suppressor mechanism (Jin and White, 2008). However, the autophagic response recycling proteins and cellular components contributes to tumor progression as a protective mechanism against stressful microenvironmental conditions including anti-cancer therapies (Amaravadi and Thompson, 2007). It has been suggested that under hypoxia, preservation of cellular fitness by autophagy may be crucial to tumor progression and aid to preventing cancer cell death (Liu et al, 2010).

The role of autophagy in response to PDT is complex. Several studies examining autophagy in cancer indicated that process as a pro-death as well as a pro-survival pathway (Apel et al, 2008). Therefore, it has been demonstrated the pro-death role played by autophagy in PDT of HeLa cells (Panzarini et al, 2011). Andrzejak and coworkers reported both autophagy and apoptosis in PDT-treated murine leukemia cells. Autophagy was shown as a way to protecting cells from photodamage in apoptosis-competent cells, by its ability to repair photodamaged cellular components (Andrzejak et al, 2011). It seems possible that autophagy is initiated in an attempt to remove organelles damaged by oxidation or in order to degrade large aggregates of cross-linked proteins, reduced and photodamaged, not removed by the ubiquitin-proteasome system (Dewaele et al, 2010). Silencing of the autophagy gene designated Atg7 results in the photosensitization of cells to photodynamic effects (Separovic et al, 2010). However, the mechanisms and roles of autophagy following PDT could also differ markedly according to cell types, depending on a variety of cell characteristics, including their propensity to undergo apoptosis, and in response to agents that produce different types or locations of damage. In fact, the efficient induction of apoptosis as well as autophagy and non-apoptotic cell deaths (e.g., necrosis), or a combination of the three mechanisms, is dependent on PS, PDT dose, PS intracellular localization, cell metabolic potential and cell genotype (Dini et al, 2010). Besides, autophagy can occur independently of apoptosis in PDT protocols, and appears to play a pro-survival role in apoptosis competent cells, and a pro-death role in apoptosis incompetent cells (Reiners et al, 2010).

After initiation of autophagy, the cytosolic autophagy protein LC3-I binds to phosphatidylethanolamine to give rise to LC3-II, which associates with autophagosomal membrane (Kabeya et al, 2000). Regardless of its function as survival or death, the processing of LC3 appears to occur in all of the cell types that have been examined following PDT (Buytaert et al, 2006; Kessel et al., 2006; Xue et al, 2007).

Exposure of a photosensitizing agent to light at a wavelength corresponding to an absorbance band leads to a photophysical reaction resulting in the release of various reactive oxygen species (ROS) which can evoke autophagy (Scherz-Shouval et al, 2007). The first line of defense against ROS can be rapidly overwhelmed during PDT, leading to oxidative stress and progressive failure of cellular machinery. In mammalian cells, the autophagy-lysosomal system represents a major proteolytic system for the clearance of ROS-damaged organelles and irreversibly oxidized cytosolic proteins (Nyström, 2005). Although the molecular mechanisms by which ROS modulate autophagy are not fully understood, the type of ROS, degree of oxidative injury and the molecular targets involved can all affect the outcome of PDT. Mitochondrial and ER-localized sensitizers cause selective photodamage to some

proteins (i.e., Bcl-2, Bcl-x, mTOR) involved in the autophagic process (Reiners et al, 2010). The pro-autophagic protein beclin-1 is known to bind to Bcl-2 (Pattingre et al, 2005). Bcl-2 is photodamaged by many PSs commonly used in investigational and clinical PDT studies (Kim et al, 1999; Kessel and Castelli, 2001). Loss of Bcl-2 function could release beclin-1 protein thus leading to the initiation of autophagy (Dewaele et al, 2010).

Interestingly, it has been demonstrated that PDT resulted in overexpression of HIF1α (Ferrario et al, 2000; Agostinis et al, 2011). HIF-1 is a master player in the adaptive response to hypoxia and influences the transcription of hundreds of genes, including the BH3-only proteins BNIP3 and BNIP3L, two pro-autophagic proteins. Thus, HIF-1-mediated induction of these BH3-only proteins can free beclin-1 from Bcl-2/Bcl-X, hereby stimulating autophagy (Bellot et al, 2009).

Within the solid tumor, where the oxygen concentration is very diverse ranging from normoxia (2–9%) to mild hypoxia (2–0.02%) to severe hypoxia (or anoxia, <0.02%), the induction of autophagy can act toward pro-survival as well as pro-death depending on the severity of the hypoxic status. Under mild hypoxia, autophagy acts as a pro-survival mechanism, since the knockdown of Atg5 or beclin-1 results in increased cell death (Bellot et al, 2009). Under severe hypoxic conditions and in the presence of additional intrinsic and extrinsic insults, like loss of tumor suppressor genes, nutrient restriction and a low pH, the induction of autophagy may exacerbate pro-inflammatory necrotic cancer cell death (Mazure and Pouyssegur, 2010).

Given the pro-survival role of autophagy, PDT-mediated vascular damage might contribute to tumor cell adaptation in the face of a hostile hypoxic environment, and enhance the resistance of surviving cancer cells to subsequent PDT. Although this has not yet been validated experimentally in vivo, it seems possible that combining PDT with an inhibitor of autophagy might result in a better therapeutic outcome (Reiners et al, 2010).

Cytoprotective Mechanisms of Resistance

The resistance of cell tumors is defined as the lack of sensibility or response to anticancer treatments. If 100% of tumor cells survive after the action of a cytotoxic agent, then it can says that there is a complete resistance. If less than 100% of cells survive, then partial resistance or partial sensitivity could be stated (Solyanik, 2010).

The resistance, independently of the mechanisms that produced it, can be classified in innate or acquired. Innate resistance is the one that a tumor presents without a previous contact with the treatment, while the acquired one appears after contact with the treatment (Liu, 2009). The induction of cellular resistance had been employed to study the resistance mechanisms to antineoplastic treatments, including PDT. The exposition to high doses of PDT is a strong selective pressure that allows survives only to more resistant cells. It is possible to obtain resistant cells to PDT increasing drug dose (Mayhew et al, 2001), drug exposure time (Sinhg et al, 1991) or light dose (Casas et al, 2006; Milla et al, 2011). Repetitive cycles of treatment and cell growth can be performed with the aim to amplify the biochemical changes associated with cell resistance and thus identify potential selective targets on the survival cells. Phenomenon of tumor resistance has been firstly found in the studies of antibacterial therapy, but nor anticancer one. The history of these studies began

from 1943, after the publication of the work of Luria and Delbruck, where it was shown that resistance of bacteria to phagocytosis result from the genetic mutations (Luria and Delbruck, 1943). In 1979, Goldie and Coldman, whose research was based on clonal theory of tumor origin and who adapted the results of studies of prokaryotic cell resistance, created the basis of clonal-selection concept of drug resistance of tumor cells. In the frame of this concept, the high frequency of genetic mutations in tumor cells leads to the appearance of different tumor clones possessing various sensitivities to the action of damaging agents. Selection factors such as cytotoxic agents cause the death of the cells that are sensitive to their action, but exert no cytotoxic effect on resistant cells (Goldie and Coldman, 1949). That results in the considerable changes of tumor cellular composition towards domination of resistant subpopulations (Solyanik, 2010).

Further to the resistance intracellular mechanisms, the tumor resistant is currently studied considering the whole tumor, possessing organ structure with components of extracellular matrix and vasculature (Hazlehurst et al, 2003). The intracellular mechanisms of resistance to PDT are related to the diminution of the intracellular accumulation of the photosensitizer, an increased inactivation of oxygen reactive species by antioxidant detoxifying enzymes, a misbalance of apoptosis proteins, the activation of heat shock proteins and the alteration of adhesion proteins. The extracellular mechanisms of resistance to PDT are related with the extracellular matrix, the vasculature and the immune system of the patient (Figure 2).

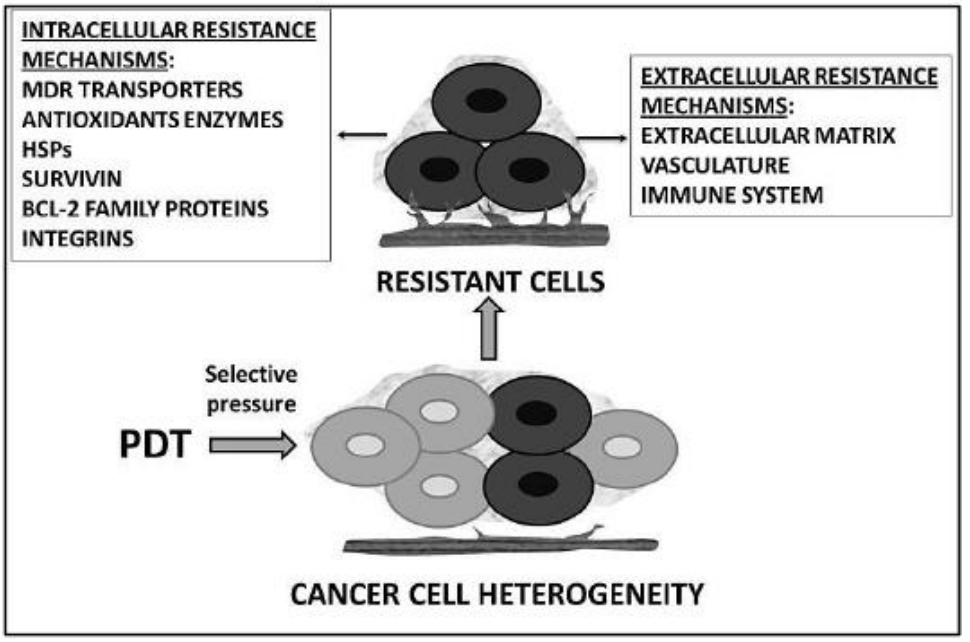

Figure 2. Cancer cells resistance mechanisms to Photodynamic Therapy. The mechanisms of resistance to Photodynamic Therapy are classified in intracellular and extracellular. The intracellular mechanisms include the diminution of the cell accumulation of the photosensitizer, the increased inactivation of oxygen reactive species by antioxidant detoxifying enzymes, the misbalance of apoptosis regulator proteins, the activation of heat shock proteins and the alteration of adhesion proteins. The extracellular mechanisms are related with the extracellular matrix, the vasculature and the immune system of the patient. HSP: Heat Shock Protein; MDR: Multidrug Resistance; PDT: Photodynamic Therapy.

Multidrug Resistance Proteins

ATP-binding cassette (ABC) transporters are a family of proteins that mediate the multidrug resistance (MDR) due it acts like ATP-dependent drug efflux pumps. These proteins are expressed in some normal tissues like epithelium of the kidney, pancreas, liver, lung and gastrointestinal tract, playing a role in the detoxification of the organs. ABC transporters are over expressed in malignant cells and pump anticancer drugs out of the cell, resulting in lack of intracellular levels of the drug necessary for effective therapy, been responsible for MDR. Various transport proteins of the ABC family have been characterized and include, for example, P-glycoprotein or multidrug resistance protein 1 (MDR-1), multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP) (Ullah, 2008).

BCRP was first discovered in doxorubicin-resistant breast cancer cells. Since the same transporter has also been found in the human placenta as well as in drug-resistant cancer cells selected in mitoxantrone, the transporter was also called ABCP or MXR1. Another alternative name to this protein frecuently employed is ABCG2. ABCG2 transporter regulates the cellular accumulation of porphyrin derivatives in cancer cells and thereby affects the efficacy of PDT and photodynamic diagnosis. ABCG2 transported protoporphyrin, hematoporphyrin and pheophorbide in an ATP-dependent manner. The activity of porphyrin efflux can be affected by genetic polymorphisms in the ABCG2 gene. Also, the oxidative stress caused by PDT can induce the transcription of the ABCG2 gene via the nuclear factor (erythroid-derived 2)-like 2 protein (Nrf2), an NF-E2-related transcription factor. The transcriptional activation and/or genetic polymorphisms of the ABCG2 gene in cancer cells may affect the responses of the patients to PDT. Protein kinase inhibitors, including imatinibmesylate and gefitinib, are suggested to potentially enhance the efficacy of PDT by blocking ABCG2-mediated porphyrin efflux from cancer cells (Ishikawa et al., 2010).

Antioxidant Enzymes

PDT produced cell death by generation of ROS. The further photodamage is well known to be antagonized by cellular antioxidant defense mechanisms, such as the glutathione system, catalase and superoxide dismutase (Casas et al, 2011). Several in vitro studies of PDT descript the relationship between the action of antioxidant enzymes and the survival of cancer cells.

Glutathione peroxidases are enzymes that catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH), a thiol-containing tripeptide (Margis, 2008). Thus, it is highly implicated in the cellular defense against xenobiotics and naturally occurring deleterious compounds (Locigno and Castronovo, 2001). The GSH content of cancer cells is relevant in regulating the resistance to treatments (Estrela et al, 2006). In human breast cancer cells, it was observed that when cells were transfected with the glutathione peroxidase gene, they were protected from PDT damage due to removal of lipid hydroperoxides in living cells after $^1O_2$ exposure (Wang et al, 2001).

Catalases catalyze the decomposition of hydrogen peroxide to water and oxygen (Alfonso-Prieto et at, 2009). PDT-induced apoptosis was impaired by addition of an

exogenous recombinant catalase analog that was specifically designed to enter cells and more efficiently localize in peroxisomes, organelles where catalases are founded (Price et al, 2009).

Superoxide dismutases (SODs) are enzymes that eliminate superoxide radicals and thus protect cells from damage induced by free radicals. The active superoxide production and low SODs activity in cancer cells may render the malignant cells highly dependent on SODs for survival and sensitive to inhibition of this (Huang et al, 2000). Human leukemia Jurkat cells over expressing manganese SOD were protected from the apoptosis initiated by PDT employing the photosensitizer phthalocyanine Pc 4 (Dolgachev et al, 2005).

Heat Shock Proteins

Heat shock proteins (HSPs) are highly conserved proteins that are induced by cellular stress signaling and play a major role in cytoprotection. HSPs have antiapoptotic action and regulate the degradation of damaged proteins after the injury to cancer cells. The overexpression of numerous HSPs is correlated with a poor prognosis in cancer cells (Thomas et al, 2005). PDT induce stress responses, promoting repair or tolerance of damage (Oleinick et al, 2002), which can be related to the appearance of resistance. Several studies have shown that HSPs are induced after PDT treatment.

PDT resistant variants of colon cancer cells and fibrosarcoma PDT resistant cells presented a higher expression of HSP60 (Hanlon et al, 2001). Stable transfected cells with HSP27 complementary DNA showed an increased survival to PDT suggesting that this protein plays a role in the resistance to PDT. PDT also induces the expression of HSP34, HSP70, HSP90 and HSP110 (Gomer et al, 1991; Curry and Levy, 1993; Gomer et al, 1996). All these protein stress have been involved in defending PDT damage.

Instead the action in cell protection after anticancer treatments, some HSPs function as endogenous adjutants that stimulate the adaptive immune response PDT (Jalili et al, 2004; Korbelik et al, 2005).

Survivin

Survivin is a protein of the inhibitor of apoptosis proteins (IAPs) family. Survivin regulates two essential cellular processes: inhibits apoptosis and promotes cell proliferation. Mechanisms of apoptosis inhibition mediated by survivin include the sequestration of procaspases 3 and 7, Smac/Diablo (Yamamoto et al, 2008) and AIF (Liu et al, 2004).

Although expressed at high levels during fetal development, survivin is rarely expressed in normal healthy adult tissues. It is however, upregulated in the majority of cancers. Because of this up regulation in malignancy and its functional involvement in apoptosis as well as proliferation, survivin is currently attracting considerable interest both as a potential cancer biomarker and as a new target for cancer treatment. Moreover, in various tumors, high survivin levels are correlated with poor prognosis, decreased apoptosis, increased angiogenesis, chemoresistance (Altieri, 2003; Zhen et al, 2005) and radio resistance (Rodel et al., 2005) in cancer cells.

It has been shown that PDT increased the expression and phosphorylation of survivin in cancer cells and that the manipulation of the anti-apoptotic pathway maintained by survivin may enhance PDT mediated cancer therapy (Ferrario et al, 2007). We have demonstrated a specific role for survivin in modulating PDT-mediated apoptotic response using a DNA vector-based siRNA, which targets exon-1 of the human survivin mRNA to silence survivin expression (Croci et al, 2008; Cogno et al, 2011). Also, in our model of squamous carcinoma cells resistant to PDT, overexpression of phospho-survivin was observed (Milla et al, 2011). Therefore, survivin has shown to be involved in resistance to PDT, and at the same time, as a target for PDT.

Bcl-2 Family Proteins

The Bcl-2 family of proteins governs whether a cell continues to live or instead commits to death through the mitochondrial apoptotic pathway. The family is divided into two functional groups: i) antiapoptotic members: Bcl-2, Bcl-xl, Bcl-w and Mcl-1 and ii) proapoptotic members including Bax and Bak, as well as the BH3-only subfamily (Bim, Bid, Bad, Bik, Bmf, HRK, NOXA and PUMA) (Chipuk et al, 2010). The influence of the balance between the pro- and anti-apoptotic members of the Bcl-2 protein family significantly influences the susceptibility of cancer cells to apoptosis following PDT (Nowis et al, 2005). Activation of anti-apoptotic Bcl-2 proteins has been early observed after PDT-treatment, thus being a mechanism supposed to be altered in PDT resistant cells. The reduction of Bcl-2 protein level by antisense Bcl-2 oligonucleotides sensitizes tumor cells to PDT-induced apoptosis (Srivastava et al, 2001). Accordingly, the transfection of tumor cells with Bcl-2 genes has been shown to protect cells from PDT-induced apoptotic death (He et al, 1996; Carthy et al, 1999). Additionally, in some other systems, Bcl-2 has been shown to undergo rapid phosphorylation, thereby restricting premature apoptosis of photodamaged cells (Vantieghem et al, 2002). In the Bax-negative prostate cancer cells, no cytochrome c release was observed indicating that Bax expression is necessary for the early steps in mitochondria-mediated apoptosis (Usuda et al, 2002).

Extracellular Matrix and Cell Adhesion

The extracellular matrix (ECM) is a complex network of macromolecules secreted by the cells. It is composed of carbohydrates and proteins such as fibronectin, vitronectin, laminin and collagen. Integrins are intermembrane proteins that connect the cells with the ECM proteins. In addition to their adhesion function, integrins mediate signaling from the extracellular space into the cell through integrin-associated signaling and adaptor molecules such as FAK (focal adhesion kinase), ILK (integrin-linked kinase), PINCH (particularly interesting new cysteine-histidine rich protein) and Nck2 (non-catalytic (region of) tyrosine kinase adaptor protein 2). Via these molecules, integrin signaling interacts with receptor tyrosine kinase signaling to regulate survival, proliferation, migration and differentiation (Hehlgans et al, 2007). Integrin-associated signaling renders cells resistant to genotoxic anti-

cancer agents like ionizing radiation and chemotherapeutic substances, through the survival via of PI3K/Akt (Velling et al, 2004) and the inhibition of caspase-8 (Estrugo et al, 2007).

Alterations in ECM and down regulation and damages in the cell adhesion proteins caused by PDT have been described for several authors (Rousset et al, 1999; Uzdensky et al, 2004; Buytaert et al, 2007; Casas et al, 2008), although the implication of this event in tumor resistance remains to be elucidated. Our group has demonstrated that cells of human squamous skin carcinoma resistant to photodynamic treatment increased their levels of integrins of the type β1 with the resistant grade (Milla et al, 2011).

PDT Triggers Dual Outcome: The "Yin and Yang" of Tumor Microenvironment

Over the past decades, research into the complex and dynamic "tumor micro-environment" (TME) has been significantly improved. The TME is currently defined as a mixture of tumor cells, that make up the parenchyma, and extracellular matrix (ECM) molecules, endothelial cells (EC), fibroblasts, immune cells such as macrophages, smooth muscle cells (SMC) and other types of cells, that constitute the stroma (Ungefroren et al, 2011). Aggressive tumors are associated with an activated environment rich in growth factors, chemokines, and proteolytic enzymes which convert stromal cells into promoters of tumor growth by enhancing angiogenesis, tissue breakdown, and tissue remodeling (Gomer et al, 2006).

Angiogenesis plays an essential role not only in the physiological formation and maintenance of vessels, but also in pathological conditions that range from tumor growth to wound healing (Drevs et al, 2002). Several steps are involved in the successful formation of new blood vessels including the stimulation of EC by growth factors, subsequent degradation of the ECM by proteolytic enzymes followed by invasion, migration, and proliferation of EC, and finally the formation of new capillary tubes (Kerbel et al, 2002). The initiation of angiogenesis, the angiogenic switch, is dependent on a dynamic regulation between pro-angiogenic and antiangiogenic factors in the immediate environment of EC (Asano et al, 1995). A positive balance in favor of angiogenic factors leads to new vessel formation, whereas the prevalence of anti-angiogenic factors shifts the equilibrium to vessel quiescence or even to vessel regression (Brekken et al, 2000). Therefore, if these angiogenic signals are blocked, angiogenesis should be inhibited and tumor growth suppressed indirectly. The next part of the chapter will focus on how PDT modulates both cellular and non-cellular constituents of the TME in order to trigger pro- or anti-angiogenic signals.

Angiogenesis as the Growing Tumor's Favorite: PDT Implications

Angiogenesis is required for tumor growth in both primary and metastatic sites (Folkman, 1995), because neovasculature is important in the supply of nutrients and oxygen to tumor cells. Therefore, through damage to these vessels, tumors can be essentially starved to death.

PDT targeted to neovasculature may cause tumor regression through the cutting off of nutrient and oxygen supplies to tumor tissues due to hemostasis (Kurohanea et al, 2001). PDT is an efficient method of killing cells that are exposed to appropriate photosensitizers and corresponding laser light. The preference of vascular versus cellular targeting of PDT treatment is highly dependent upon the relative distribution of the photosensitizer in the vascular and cellular compartments and can also be effectively manipulated by varying the drug and light intervals (DLI). Initially after photosensitizer is administered, the drug is confined within the tumor vasculature and employing a short DLI largely damages the tumor vessels. However, during longer DLI the photosensitizer diffuses out from the blood vessels into the tissue, and accumulates in the tumor cellular compartment and the subsequent light irradiation targets the cells and causes tumor cytotoxicity (Bhuvaneswari et al, 2008).

Photodynamic perturbation of tissue microcirculation was first reported in 1963 (Castellani et al, 1963). Vascular damage has been implicated as the primary antitumoral effect in PDT with various photosensitizers (Fingar et al, 1996, 1999, 2000; Chen et al, 2002b, 2006; Olivo et al, 2006). It is believed that the circulating photosensitizer in the plasma generates cytotoxic reactive oxygen species, which leads to primary vascular damage that result in tumor necrosis. It has also been acknowledged that targeting tumor vasculature proves to be a promising approach in cancer treatment (Dolmans et al, 2002). The effect of PDT on implanted tumor with the PDT regimen designed to induce hemostasis resulted in drastic tumor regression and curing of mice. These results indicate that tumor cells could not survive under the hemostasis of neovasculature. In order to optimize drug delivery, cationic molecules have been shown to be useful for enhancing interaction of the photosensitizer entrapped in liposomes with the vascular EC of angiogenic vessels. It would be expected that cationic molecules would adhere to the anionic plasma membrane by electrostatic interaction (Ran et al, 2002). Moreover, the PDT-mediated anti-tumor effect of benzoporphyrin derivative monoacid ring A (BPD-MA) entrapped in polycation liposomes (BPD-MA PCLs) depended on the angiogenic vessel-damage effect, because BPD-MA PCLs induced apoptosis of EC (Takeuchi et al, 2003a, 2003b). These results suggest that specific destruction of angiogenic vessels in PDT has a potent anti-tumor effect without severe side effects (Shimizu and Oku, 2004). PDT against EC of the tumor neovasculature may be expected to be effective for various tumors, including drug-resistant ones. Furthermore, the doses can be reduced compared with those for tumor targeting PDT and the treatment can be finished in a shorter time. Thus, the present modality would be quite beneficial for patients (Kurohanea et al, 2001). Chen and coworkers observed that inactivation of vessels in the tumor center occur earlier than in the tumor periphery and surrounding tissue. This difference in response implies that vasculature in the tumor center is more sensitive to PDT-induced vascular damage than is vasculature in the tumor periphery and surrounding tissue, and this might lead to selective damage to tumor vasculature. Complete tumor cure was obtained after this PDT regime (Chen et al, 2002a).

The recognition that tumor vascular damage is largely responsible for tumor destruction after in vivo PDT encourage tumor vasculature to be a promising target for PDT (Henderson and Dougherty, 1992; Fingar, 1996; Veenhuizen et al, 1997; Zilberstein et al, 2001; Chen et al, 2002a). Compared with the direct cell-killing approach, this antivascular strategy has the potential to kill many more tumor cells because of the crucial role of blood supply in the survival, growth and spread of solid tumors. In 1998, Gomer et al. showed that bovine EC were significantly more sensitive to PDT with porfimer sodium than SMC or fibroblasts from

the same species. Exponentially growing EC were significantly more sensitive than similarly proliferating tumor cells, and the difference in sensitivity was accompanied by greater PS accumulation in the former. Endothelial cell responses to sublethal doses of PDT may also contribute to vascular changes observed in tissue (Agostinis et al, 2011). This result is in agreement with those of our preliminary evidences, in which microvascular human cell line has shown increased photosensitivity to methyl-ALA PDT respect to human colon cancer cells (data not published).

As described above, extensive evidence has shown that PDT targeting angiogenic vasculature effectively suppressed tumor growth compared with PDT targeting the tumor itself. Therefore, selectively damaging existing microvasculature and preventing the formation of new blood vessels would improve treatment efficacy.

Although PDT has been approved by FDA and regulatory agencies worldwide for several oncologic and non-oncologic conditions (Peng et al, 1997; Dougherty et al, 1998) tumor recurrence is still the major limitation of the modality (Ji et al, 2006; Agostinis et al, 2011). Non-homogeneous light distribution, incomplete photosensitizer dosage and tissue/tumor dynamics are some of the factors that impose constraints on the efficacy of PDT. Oxygen is one of the three key parameters for PDT efficacy and PDT induced highly toxic singlet oxygen and oxygen radicals can react with cellular components to cause cell death. Moreover, as PDT induced oxidative stress can cause hypoxic condition in the surviving tumor cells due to vascular damage and photochemical oxygen consumption (Peng et al, 1996; Peng and Nesland, 2004), it can elicit the expression of angiogenic growth factors and cytokines as an adaptive response (Dougherty et al, 1998; Gollnick et al, 2003). The relationship between PDT and angiogenesis was first established by Ferrario and colleagues (Ferrario et al, 2000). When there is a hypoxic condition in tumor tissue, tumor cells receive this signal, which is often mediated by hypoxia inducible factor-1 (HIF-1), to promote an angiogenic switch and induce angiogenesis (Hanahan and Folkman, 1996; Carmeliet et al, 1998; Bergers and Benjamin, 2003). Angiogenesis is regulated by numerous cytokines including pro-angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietin, basic fibroblast growth factor (bFGF), placenta-like growth factor (PLGF), platelet-derived growth factor (PDGF), and IL-8, as well as the anti-angiogenic factors angiostatin, endostatin, thrombospondin, a tissue inhibitor of metalloproteinase (TIMP), and so on (Relf et al, 1997). These angiogenic factors are produced by not only tumor cells but also by stromal cells. In tumor growth, pro-angiogenic factors predominantly exist in an angiogenic site and stimulate vascular EC through their receptors (Relf et al, 1997), which acquire specific characteristics to constitute new vessels.

Hypoxia results in stabilization of HIF-1α, which helps to restore oxygen homeostasis and stimulates cell survival processes by inducing glycolysis, erythropoiesis, apoptotic inhibition and angiogenesis (Brahimi-Horn and Pouyssegur, 2005; Carmeliet et al, 1998; Semenza et al, 1994). Previous studies have thus far concentrated the effects of PDT-induced hypoxia on expression of pro-angiogenic factors as well as their relevance to PDT efficacy (Ferrario et al, 2000; Zhou et al, 2005). This transcription factor (HIF) may make the cell proliferation for survival adaptation with resistance to PDT, a possible mechanism contributing to less PDT efficacy and/or tumor recurrence. Several studies have shown that hypoxia can activate phosphatidylinositol 3-OH kinase, one of the critical downstream mediators of the tyrosine kinase signaling pathway that regulates cell proliferation and suppression of apoptosis (Chen et al, 2001; Lee et al, 2004). Hypoxia-induced HIF-1α

expression attenuates PDT efficacy through probably not only induction of angiogenesis, but also increase in cellular resistance to PDT. Ji and coworkers showed that overexpression of HIF-1α by $CoCl_2$ under a normoxic condition may render the Het-1A cells resistant to ALA–PDT (Ji et al, 2006). These results are in good agreement with the report by Koukourakis et al. who have shown that high expression of HIF-1 was associated with a low complete response rate of human early esophageal cancer after hematoporphyrin derivative-based PDT (Koukourakis et al, 2001). The present finding may have important impact on understanding of clinical PDT outcome.

Regarding tumoral cell response, PDT application leads to activation of multiple signaling pathways (Gomer et al, 2006; Bhuvaneswari et al, 2009b). In this chapter we have focused attention on vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs).

VEGF is considered one of the most important growth factors regulating physiological and pathological angiogenesis (Ferrara, 2004). VEGF expression is regulated by a variety of host stimuli including cytokines and cellular or tissue stress. Hypoxia also serves as a strong stimulus for VEGF expression via activation of the HIF-1 transcription factor. Roberts and Hasan (Roberts and Hasan, 1993) were the first to report on a connection between VEGF expression and photosensitizer uptake in tumors. Ferrario et al have shown that PDT induces expression of the transcription factor HIF-1α as well as the HIF-1α target gene VEGF in treated tumors (Ferrario et al, 2000; Ferrario and Gomer, 2006). Holding a similar view, Gomer and his colleagues also reported that HIF-1α and VEGF expression within the tumor microenvironment are modulated by PDT fluence rate. High fluence rates can deplete tumor oxygenation and decrease the effectiveness of PDT (Gomer et al, 2006). Nowak-Sliwinska and her colleagues also reported that the angiogenesis process resulting from Visudyne-PDT involves vascular neoformation concerning among other growth factors, VEGF and VEGF signaling (Nowak-Sliwinska et al, 2010). In response to therapy, accumulating data suggest that upregulation of VEGF create enhanced environment for tumor recurrence (Uehara et al, 2001; Yee et al, 2005; Ferrario and Gomer, 2006; Bhuvaneswari et al, 2007a; Bhuvaneswari et al, 2009b). Matrix metalloproteinases (MMPs), which are zinc dependent endopeptidases and consist of about twenty members, play an important role in tumor metastasis and angiogenesis. Several of these enzymes are found within the tumor microenvironment and are involved in tumor angiogenesis and invasion (Bergers et al, 2000; Coussens et al, 2002). The in-vivo activity of MMPs is regulated in part by endogenous tissue inhibitors of MMPs or TIMPs. Cytokines, growth factors, oncogenes, and reactive oxygen species are among the stimuli that activate MMP transcription. In the case of PDT responsiveness, MMP-9 may play an important role in modulating the tumor microenvironment (Gomer et al, 2006). Ferrario and coworkers have documented that treatment of mouse tumors with PDT induces strong expression of MMP-9 and the extracellular MMP inducer (EMMPRIN) along with a concomitant decrease in expression of TIMP-1 (Ferrario et al, 2004). MMP-9 was expressed by host cells within the tumor microenvironment but not by tumor cells. There is evidence that MMP-9 contributes to tumor angiogenesis by assisting in the formation of capillary networks and recruitment of VEGF in the tumor microenvironment as well as by promoting the invasion of the extracellular matrix by EC (Chantrain et al, 2004; Jodele et al, 2005).

Tumor hypoxia is a therapeutic concern as it reduces the effectiveness of radiotherapy, chemotherapy and PDT, thus leading to angiogenesis and tumor metastasis. Numerous studies have documented the use of anti-angiogenic agents along with conventional cancer treatments

to enhance the antitumor response. In a similar way combining angiogenesis inhibitors with PDT in preclinical studies has clearly demonstrated increased therapeutic efficacy. However, blocking one or two molecular pathways might not provide optimal results, as multiple pathways are involved in the migration and proliferation of tumor cells. Therefore, in order to achieve complete and effective tumor response, using combination therapy and targeting different molecular pathways without increasing toxicity, could be an attractive therapeutic strategy. The use of agents that enhance the efficacy without increasing the normal tissue effects of PDT, thereby improving the therapeutic index, will represent a major focus of clinical research going forward. Antivascular effects of PDT can be further potentiated by antiangiogenic or antivascular drugs (Ferrario et al, 2000; Bhuvaneswari et al, 2009a) or monoclonal antibodies targeting factors such VEGF promoting neovascularization (Bhuvaneswari et al, 2007b, 2011) significantly improving tumor growth control after PDT. Additionally, a positive correlation exists between expression/activation of MMPs and tumor angiogenesis, growth, invasion, and metastatic potential (Bergers and Coussens, 2000; Nelson et al, 2000). For this property, a combining PDT with agents that induce pharmacological inhibition of MMPs can selectively increase in vivo PDT tumoricidal activity (Ferrario et al, 2004). Finally, the present chapter provides the evidence that overexpression of HIF-1α can reduce photosensitivity of PDT in vitro. Thus, PDT in combination with anti-HIF-1α treatment may enhance PDT efficacy.

Tumor Surrounding Stroma as an Active Player of Tumor PDT Response

In 2008, Gatenby and Gillies proposed that tumor development is associated with a differential protein expression profile which initiates a non-physiological molecular crosstalk between the transformed epithelial cell and the surrounding stroma. This reciprocal interaction of cells generates interdependent cell types and gradually impairs the integrity and barrier function of the microenvironment which culminates in its permanent remodeling (Gatenby and Gillies, 2008). This remodeling relates to an increased number of fibroblasts, enhanced capillary density and deposition of a new ECM rich in type-1-collagen and fibrin. Macrophages are also recruited in response to the healing process generated by the tumor (Gout and Huot, 2008). Recently, Hanahan et al postulate that the integration of cues from all components of the TME is crucial to regulate cell behaviour and also define cancer phenotype (Hanahan and Weinberg, 2011). The tumor microenvironment affects angiogenesis by interfering with the signaling pathways required for cell recruitment and vascular construction (Ungefroren et al, 2011), considering that blood vessel formation by angiogenesis requires the tight control and coordination of EC behavior (Herbert and Stainier, 2011).

Endothelial Cell

Angiogenic endothelial cells are believed to express several specific molecules, which are known or unknown, in comparison with normal endothelial cells. Therefore, these distinct marker molecules may provide active targeting guides for anti-neovascular therapy. Many

reports cited in this chapter directly implicate the endothelium as a primary target for PDT in vivo; this stimulated research into the relative sensitivity of EC to PDT and the responses of EC that could initiate the various phenomena at the vessel level.

The formation of new microvessels are mediated by the endothelial cell adhesion molecules (CAMs) such as integrins, selectins, cadherins and immunoglobulins (Hsu et al, 2007) and the lack of appropriate cell contacts may even lead the EC to programmed cell death (Meredith et al, 1993). Hypericin-PDT induces extensive vascular and cellular damage, due to which an expected downregulation of CAMs, cadherin 5, collagen α1 and collagen α3, integrin αV and integrin β3 was observed. However, when the PDT induced damage diminished, upregulation of these molecules were noted (Bhuvaneswari et al, 2008). Additionally, the expression levels of the adhesion molecule ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1) were down-regulated in EC after PDT (Volanti et al, 2004) (Figure 3).

Activation of EC is initiated with binding of the pro-angiogenic factors VEGF, bFGF, PDGF and so on (Relf et al, 1997) to their receptors expressed on the EC followed by transduction of the angiogenic signaling in the cells (Shimizu and Oku, 2004).

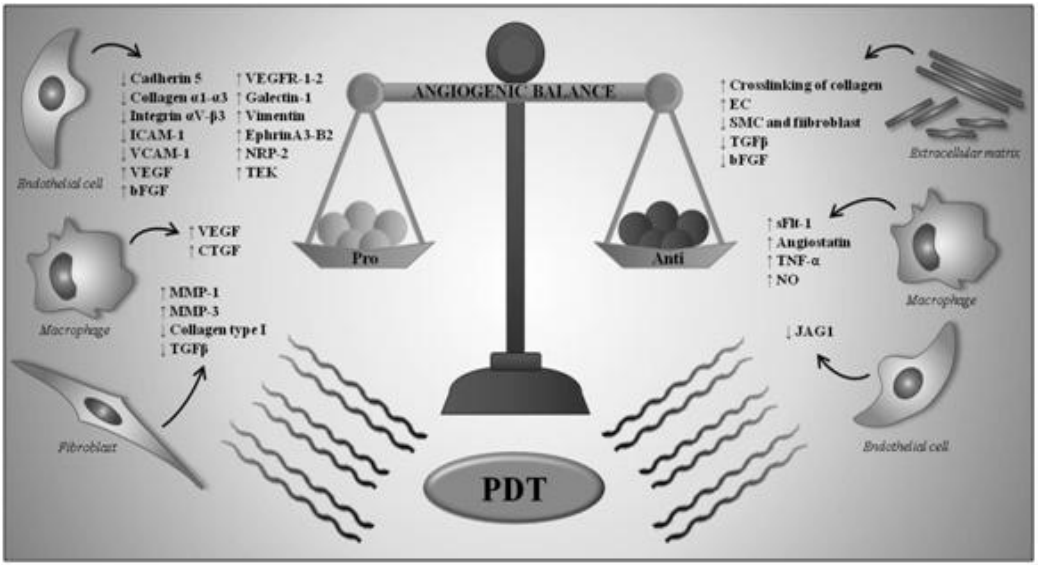

Figure 3. Active role of tumor stroma in molecular and cellular profiling of angiogenesis induced by Photodynamic Therapy. PDT-mediated oxidative stress can act as an angiogenic switch that ultimately leads to neovascularization and tumor recurrence. This angiogenic switch depends on a dynamic regulation between pro and anti-angiogenic factors in the immediate environment, produced by not only tumoral cells but also by stromal components of their microenvironment. Anyway, differential expression of genes involved in the angiogenesis pathway is finally conditioned by photosensitizer, drug and light interval, cell behavior and so on, which they will be further elucidated. bFGF: basic fibroblast growth factor; CTGF: connective tissue growth factor; EC: endothelial cell; ICAM-1: intercellular adhesion molecule 1; JAG-1: Jagged-1; MMP-1: matrix metalloproteinase 1; MMP-3: matrix metalloproteinase 3; NO: nitric oxide; NRP-2: neurophilin 2; sFlt-1: soluble fins-like tyrosine kinase 1; SMC: smooth muscle cell; TEK: tyrosine *kinase endothelial; TGFβ:* transforming growth factor beta; TNF-α: tumor necrosis factor alpha; VCAM-1: vascular cell adhesion protein 1; VEGF: *vascular endothelial growth factor; VEFGFR-1-2: vascular endothelial growth factor receptor 1-2.*

Molecular profiling by quantitative real-time PCR demonstrated the spiking of the angiogenic growth factors VEGF and bFGF as well as the upregulation of VEGF receptors. VEGF and VEGF receptors play a role in the PDT induced angiogenesis response. Interestingly, gene expression in PDT-treated chicken embryo chorioallantoic membrane of both VEGF and bFGF is rapidly induced and already measurable after 6 h, indicative of the ''explosive'' angiogenic response to the PDT induced stress (see Figure X). After 24 and 48 h, the level of the angiogenic growth factors returns to control levels, but the pro-angiogenic condition is than maintained by enhanced expression of the growth factor receptors. The other tested VEGF receptors, VEGFR-1 and VEGFR-2, were upregulated later (24 h after PDT) and stayed high (Nowak-Sliwinska et al, 2010). Deiniger et al also reported induction of VEGF by treated-EC using hypocrellin-A and –B as photosensitizer (Deininger et al, 2002).

Galectin-1 and vimentin are widely used as other activation markers of angiogenic blood vessels. Galectins are a group of mammalian beta-galactoside binding proteins with diverse functions that are not yet fully defined. It has been shown that galectins have intracellular regulatory roles in RNA splicing, act to inhibit or induce apoptosis, stimulate cell proliferation and differentiation, and regulate the cell cycle. Galectin-1 was previously found to function as the endothelial receptor for anginex, a novel peptide angiogenesis inhibitor (Thijssen et al, 2006). Also vimentin was studied since it was reported to be overexpressed in angiogenic endothelium (van Beijnum et al, 2006). Interestingly, vimentin cleavage has been reported in response to various inducers of apoptosis, such as ionizing radiation (Prasad et al, 1998) and PDT (Belichenko et al, 2001). Since vimentin is overexpressed in tumor EC (van Beijnum et al, 2006), vimentin cleavage may be used as a diagnostic tool to assess therapeutic efficacy of PDT as well as apoptotic changes in certain tumors vessels treated with PDT. Nowak-Sliwinska and coworkers showed that galectin-1 and vimentin are overexpressed in the newly formed vasculature in the PDT area as compared to the untreated area. These data demonstrate that the vasculature contains angiogenically-activated blood vessels, and suggests that the formation of neovasculature occurs after application of PDT (Nowak-Sliwinska et al, 2010) (Figure 3).

Significantly enhanced transcription of ephrin A3 and ephrin B2 was observed 30 days post short and long DLI PDT. These are classes of proteins that bind to the Eph receptors, a subset of the EC receptor tyrosine kinase (RTK), and promote cell attachment, cell-adhesion migration, capillary tube formation and ultimately lead to tumor angiogenesis (Cheng et al, 2002; Sawamiphak et al, 2010). The Eph receptors also regulate integrin-dependent cell adhesion through activation of the c-Jun kinase via Nck-interacting Ste20 kinase in EC (Becker et al, 2000). This further suggests that the downstream events in the ephrin signaling can lead to enhanced angiogenesis even at 30 days post PDT (Bhuvaneswari et al, 2008).

Neuropilins are widely expressed in normal mature and developing vasculature both on vascular SMC (Stephenson et al, 2002; Yang et al, 2005) and EC (Soker et al, 1997), as well as in tumor cells. Experiments suggest that neuropilins mediate pro-angiogenic effects by binding to VEGF. Neuropilin-2 (NRP-2) expression correlates with advanced tumor stage and grade (Ellis, 2006). Its upregulation at 24 h post PDT can be correlated to tumor angiogenesis and tumor progression (Bhuvaneswari et al, 2008).

The notch signaling controls multiple cell differentiation processes, including proliferation, migration, smooth muscle differentiation, angiogenic processes, and arterial-venous differentiation (Sainson and Harris, 2008). The most studied notch ligands, Delta-like 4 and Jagged1 (JAG1), competitively regulate tumor angiogenesis. Studies have demonstrated

that Delta-like 4 functions as a negative regulator of tumor angiogenesis, whereas JAG1 promotes angiogenesis (Bridges et al, 2011). Downregulation of JAG1 post PDT suggest the pro-angiogenic notch signaling cascade could be suppressed (Bhuvaneswari et al, 2008). On the other hand, TEK (tyrosine kinase, endothelial cell), also known as TIE-2, constitute a subfamily of receptor tyrosine kinase, known for its specific expression in vascular endothelial cells (Sato et al, 1998). The TEK gene was found to be upregulated 30 days post short and long DLI PDT (see Figure X). Thus it can be speculated that the expression of this tyrosine kinase would further signal the downstream pathway leading to cell proliferation, migration and survival (Loughna and Sato, 2001). These results suggest that Ang1/Tie2 signaling plays an important role in EC survival post Hypericin-PDT (Bhuvaneswari et al, 2008).

The involvement of angiogenesis in tumor growth has been very well documented in this chapter but how EC response to PDT induced tumor stimuli has been poorly reported. Relying on the hypothesis that angiogenic regulator concentration within the tumor microenvironment is a requirement on EC stimulated to enter an angiogenic program functional studies have been done. Preliminary lines of evidences from our work shown that molecules released by PDT-treated human colorectal cancer cells (SW480ADH) exposed to lethal dose of methyl-ALA-PDT (less than 35% survival) could confer a stimulation of angiogenic pattern on target endothelial cells (HMEC). The biological effects of soluble regulator resulted in proliferation, migration and capillary morphogenesis which are indispensable to angiogenesis (data not published). This observation let us in a large part propose to EC as a key TME component of tumor PDT recurrence by promoted vascular supply.

Extracellular Matrix

As angiogenesis proceeds, the extracellular matrix (ECM) serves essential functions in supporting key signaling events involved in regulating EC migration, invasion, proliferation, and survival (Davis and Senger, 2005). Interactions between cells and their ECM are tightly regulated and drive a variety of biological outcomes. This matrix is made up of carbohydrates and proteins including adhesion proteins such as fibronectin, vitronectin, laminin, tenascin, and collagen, which are secreted by surrounding cells. The molecular composition and spatial organization of the ECM can be affected by biological, chemical or physical factors. Therefore, changes brought on the ECM may influence both cell-substrate and cell-cell crosstalk (Hay, 1990).

In cancer, the composition of the ECM is often altered by factors produced by cancer and stromal cells. Matrix metalloproteinases (MMPs) are one of the most important factors involved in degradation and remodeling of the ECM, which in turn affect many aspects of tumor development such as cellular interactions and cell dissemination. Matrix invasion is a crucial prerequisite for angiogenesis and posterior metastasis. That phenomenon is dependent on the expression of CAMs and matrix degrading enzymes. The ECM also influences the bioavailability of secreted factors and regulates tumor stiffness in order to enhance cell motility and migration (Ungefroren et al, 2011).

There is insufficient published scientific evidence evaluating the effect of PDT on the ECM of the tumor microenvironment (TME). Nevertheless, the relationship between PDT and the ECM was first evaluated by Ortu et al, who proposed to PDT as a novel and beneficial strategy to inhibit intimal hyperplasia (IH) after vascular procedures. This good outcome can not be ascribed only to the photocytotoxicity, so it is suggested that changes in the crosstalk between ECM and cells could be involved (Ortu et al, 1992).

ECM components are affected by PDT even when they are not the primary targets. For this reason, several *in vitro* models have been developed to better understand the biologic consequences of PDT on it. Adili et al were the first who demonstrated that ECM-cell interactions are modulated as a result of photosensitization. ECM secreted by bovine aortic EC (EC-ECM) treated with chloroaluminum-sulfonated phthalocyanine (CASPc)-PDT modulates the function of bovine SMC and EC. When compared with untreated ECM, treated ECM significantly reduced SMC attachment, proliferation and migration. On the contrary, EC proliferation and migration were significantly potentiated after PDT of EC-ECM (Adili et al, 1996). In a later study, these investigators also observed that CASPc-PDT of 3-dimensional gel, containing collagen type-I/type-III and serum proteins, led to a reduction of invasive bovine and human SMCs and fibroblast migration (Overhaus et al, 2000; Waterman et al, 2002). Heckenkamp and coworkers also demonstrated that PDT of collagen gels using methylene blue (MB) as photosensitizer reduced non-treated fibroblast proliferation (Heckenkamp et al, 2004).

Many methods have been used in an effort to elucidate biochemical or structural changes that may occur on ECM after PDT. In this context, MMP levels were assessed in cultures of bovine SMCs on PDT-treated matrix and, in the conditioned media secreted by SMC, MMP-2 was present, which may explain in part why SMC migration was significantly reduced but not abolished (Overhaus et al, 2000). On the contrary, pro-MMP-2 and -9 levels of human SMC cultured on PDT-treated collagen gels remained unaltered, suggesting that although MMP play a role in invasive migration, PDT on collagen matrix does not affect their production or activation (Waterman et al, 2002). On other hand, several studies identified collagen matrix changes after PDT, including cross-linking, which resulted in resistance to protease digestion (Overhaus et al, 2000; Waterman et al, 2002). This effect appears to be principally mediated by free radical interactions with amino acids, which lead to conformational and other chemical changes that modify biologically active or specific binding sites of ECM proteins. Because PDT showed a similar inhibitory effect on SMC and fibroblast migration, and no significant change in the secretion of MMP was observed, it is theorized that a major contributor to the inhibition of cellular migration following PDT is ECM cross-linking (Waterman et al, 2002).

In order to further elucidate why PDT treated ECMs affect the proliferative capacity of non-treated cells, it has been investigated about the effect of PDT on two matrix-associated bioactive molecules: transforming growth factor beta (TGFβ) and basic fibroblast growth factor (bFGF). Although TGF-β is a potent inhibitor for endothelial cells proliferation (Heimark et al, 1986), it can stimulate the mitogenesis of fibroblast (Kay et al, 1998) and SMC (Berk, 2001). Moreover, TGF-β can acts as a potent chemoattractant for a variety of monocytes/macrophages and fibroblasts (Merwin et al, 1991). On the other hand, bFGF is a powerful matrix-resident mitogen and has been implicated as the major primary initiator of SMC replication after vascular injury, associated with ECM by heparin sulfate binding sites (Nabel et al, 1996). It was shown that free bFGF and also bFGF bound to the ECM in vitro

were sensitive to PDT (LaMuraglia et al, 1997). A further study demonstrated that PDT-free fibroblast placed on PDT-treated collagen matrix significantly decreased TGFβ and bFGF mRNA levels (Heckenkamp et al, 2004). In addition, levels of TGF-β could be barely detected after PDT on EC-ECM (Statius van Eps et al, 1997). That could provide a mechanism, at least in part, by which PDT of ECM diminished SMC and fibroblast proliferation but enhance EC proliferation. There is no evidence that this PDT effect would be specific to bFGF or TGFβ, thus these findings concur with prior observations that PDT affects proteins mainly by free radical affinity for the amino acids (Freeman and Crapo, 1982) (Figure 3).

These results may indicate that photosensitized ECM could act as a barrier to invasive cellular migration and recruitment of endothelial cells, thus promoting anti-angiogenic features. This is supported by an increased collagen cross-linking making it more resistant to degradation by proteases, failure to induce MMPs secretion and inactivation of matrix-associated growth factors. Overhaus et al suggest that it seems unlikely that PDT could augment the biological effect of inhibitor of MMPs, such as TIMP, because PDT has been demonstrated to inactive biologically active matrix-associated proteins by free-radical non-specific interactions (Overhaus et al, 2000).

Considering the key role of interactions between ECM with the whole TME to modulate tumor invasion and angiogenesis, it seems essential to define how PDT could influence ECM composition and proteins-associated surrounding tumor cells.

Fibroblasts

Besides the ECM, non-neoplastic cells in the TME impact on tumor cell angiogenesis (Ungefroren et al, 2011). It is now well-established that non-malignant cells of the microenvironment provide malignant cells with a set of growth factors, chemokines, and integrin ligands which stimulate cancer cell growth, migration and invasion. Similarly, the non-malignant cells are a rich source of proangiogenic factors (Gout and Huot, 2008).

Fibroblasts are the major stromal cells whose function is to synthesize ECM components like collagen and fibronectin. Fibroblasts associated with tumor cells change their phenotype towards contribute to tumor progression, now referred to as “cancer-associated fibroblasts” (CAF). In this activated state, they acquire the ability to emit paracrine signals that promote tumor growth and invasion, enhance angiogenesis and modulate tumor immunity, by producing a variety of cytokines, growth factors, ECM proteins and MMPs (Räsänen and Vaheri, 2010). By producing the two latter, they could determine the biophysical properties of the ECM, thereby indirectly serve as pathfinders for the sprouting endothelium.

It has been widely reported that normal fibroblast proved to be more resistant to PDT than tumor cells or transformed fibroblast (Uberriegler et al, 1995; Haddad et al, 1999; Melnikova et al, 1999; Tong et al, 2000; Triesscheijn et al, 2004; Zenzen and Zankl, 2004; Pittet et al, 2007; Maduray et al, 2011). Due to the importance of fibroblast collagen production, several studies investigating the effect of PDT on collagen synthesis have been carried out. In this context, heat shock protein 47 (HSP47) is known to be a chaperone specifically involved in processing collagen molecules, and it has the ability to bind to collagen type I and its precursors; furthermore HSP47 plays an active role in collagen type I

byosynthesis (Koide and Nagata, 2005). Researchers have demonstrated that sublethal and isoeffective conditions of PDT with the photosensitizer haematoporphyrin ester (HpE) (Verrico and Moore, 1997; Haylett et al, 2002), meta tetra hydroxyphenyl chlorin (mTHPC) (Verrico and Moore, 1997) or ALA (Shackley et al, 2002) on normal skin fibroblast, did not induce HSP47 expression. These investigators also observed similar results in vivo on murine skin, and absence of skin fibrotic characteristics was also reported three months after HpE-PDT (Verrico et al, 2001). Using the same protocol, in the case of hyperthermia, riboflavin (RB)-PDT or ionizing radiation (Verrico and Moore, 1997; Verrico et al, 2001), this trend was reversed. In addition, parallel work on normal skin fibroblast showed an increase in soluble collagen following hyperthermia and ionizing radiation, but not HpE PDT, correlated well with the observed previously (Haylett et al, 2002). The expression level of HSP47 and collagen type I following application of different stress conditions could depend on which particular kind of stress was applied. Based on the idea that hyperthermia, ionizing radiation and RB-PDT damage collagen so they promote its synthesis, the lack of elevation or even the down-regulation of HSP47 and collagen synthesis after PDT could be consistent with the absence of damage to collagen (Verrico and Moore, 1997).

On the contrary, Uehara et al showed that PDT using Hp oligomers as photosensitizer enhances the synthesis of collagen type I in the fibrous tissue adjacent to squamous cell carcinoma in mice, which contribute to resultant encapsulation of such tumor; however, the proliferative activity of fibroblast was not correlated with the induction of HSP47 (Uehara et al, 2007).

Moreover, it has been shown that PDT is able to enhance MMPs expression by fibroblasts. Karrer et al showed that sublethal treatment of normal dermal and scleroderma fibroblast with ALA-PDT subsequently placed on a collagen lattice, caused a marked induction of MMP-1 and -3 mRNA and protein, which are important for collagen degradation. TIMP-1 and TIMP-2 mRNA were not affected, while collagen type I mRNA expression was decreased; these features favor all together suggest a collagen reduction by fibroblast post PDT (Karren et al, 2003). In a later study, essentially the same results were obtained using ATX-S10(Na) as photosensitizer (Takahashi et al, 2006). Karrer and coworkers also presented evidence for the *in vitro* paracrine activation of MMP-1 and -3 production in dermal fibroblast. This activation is mediated by soluble factors, in particular IL-1 and TNF-α, released by ALA-PDT-treated epidermal keratinocytes (Karrer et al, 2004) (Figure 3).

It is widely recognized that TGF-β, besides its potent stimulus for fibroblasts proliferation, increases the production of collagen and other extracellular matrix proteins, inhibiting their degradation by reducing MMPs synthesis (Tjäderhane et al, 2001). Byun et al demonstrated a reduction in transcription and translation of the TGF-β mRNA and protein respectively by cultured fibroblast treated with ALA-PDT (Byun et al, 2011).

Based on above, we suggest that there is a secondary mechanism mediated by PDT on fibroblast, along with paracrine stimulation by other stromal and parenchyma cells, which could promote a microenvironment more suitable for angiogenesis and metastasis by degrading the surrounding matrix. Induction of collagen-degrading enzymes (MMPs) together with reduction of collagen production by phosensitized fibroblasts might be in part responsible for the subsequent invasion of tumor cells and recruitment of endothelial cell.

Macrophages

As already mentioned above, macrophages are classified into tumor stromal cells. Macrophages form a heterogeneous cell population belong to the mononuclear phagocyte system (Sunderkotter et al, 1994). Although the original hypotheses proposed that macrophages are involved in antitumor immunity, numerous clinical and experimental evidences indicate that macrophages promote cancer initiation and malignant progression (Qian and Pollard, 2010). Macrophages acquire specific phenotypes, depending on the environment of the tumor. The ‘‘classically-activated’’ macrophage, or M1 macrophage, displays an anti-angiogenic phenotype, and plays an important role in anti-bacterial and pro-inflammatory functions. On the other hand, ‘‘alternatively-activated’’ macrophages, or M2 macrophages, are pro-angiogenic and produce high levels of IL-10 and low levels of pro-inflammatory cytokines such as IL-6 and TNF-α (Dace et al, 2008). Macrophages and monocytes express several ‘‘scavenger’’ receptors which are membrane proteins that recognize a wide range of ligands (Freeman, 1997).

Tumor-associated macrophages (TAMs), in contrast with normal macrophages, exhibit the M2 phenotype, and thereby exhibit pro-tumoral functions. These include supporting tumor-associated angiogenesis, promotion of tumor cell invasion, migration, and intravasation, as well as suppression of antitumor immune responses (Pollard, 2004; Condeelis and Pollard, 2006).

Regarding TAMs relevance in tumor development, it has been proposed to be a “target for cancer therapy”. The Class A scavenger receptor (SRA) is a multidomain trimeric transmembrane protein largely confined to mature macrophages and therefore investigators have explored the possibility of preparing covalent conjugates between SRA ligands and PS to produce macrophage targeted PDT (Mukhopadhyay et al, 1995; Hamblin et al, 2000; Liu and Hamblin, 2005).

It has been reported release of regulators of angiogenesis following hypocrellin-A and –B PDT of human brain macrophages. The anti-angiogenic proteins sFlt-1 (soluble truncated form of Flt-1/VEGFR-1), which acts as a dominant-negative inhibitor of VEGF, and angiostatin were upregulated post-PDT. On the other hand, pro-angiogenic proteins, such as VEGF and connective tissue growth factor (CTGF) were overexpressed by macrophages under the same conditions (Deininger et al, 2002). CTGF is a 349 aminoacid protein identified as a modulator of fibroblast activity, by enhancing the expression of type I collagen, fibronectin and integrin α5 subunit, and as a promoter of angiogenesis (Lau and Lam, 1999) by binding to integrins αVβ3 that has been implicated in tumor neoangiogenesis and metastasis. This may promote the revascularization, reoxygenation, and regrowth of the tumor once the therapy concludes, although the influence of PDT-treated macrophages on angiogenesis process is not fully known due to its concomitant release of anti-angiogenic factors.

Several studies indicated that PDT-treated macrophages exert potentiated phagocytic activity, produce increased amounts of TNF-α and nitric oxide (NO) and thus exert antitumor effects (Evans et al, 1990; Yamamoto et al, 1991, 1992; Coutier et al, 1999). Experiments performed by Kawczyk-Krupka showed that ALA-PDT treated macrophages increase in reactive oxygen intermediates (ROI) and TNF-α release and decreased levels of nuclear factor κB (NF-κB), IL-1β production and NO release. Both TNF-α and NO have numerous

physiological functions and, besides direct tumoricidal action, they might affect tumour microvasculature resulting in tumour regression (Asher et al, 1987; Korbelik et al, 1998) (Figure 3).

In another context, TAMs accumulate in hypoxic areas of the tumor and are particularly associated with necrotic tissue (Murdoch et al, 2008). Macrophages response to hypoxia includes up-regulation of transcription factors HIF-1 and-2, which in turn activate mitogenic, proinvasive, proangiogenic, and prometastatic genes (Lewis and Murdoch, 2005). It can be expected that treatment known to induce hypoxia, as PDT (Schouwink et al, 2003) may impinge on the recovery of tumors. Future studies should also be accompanied by the examination of the effect of PDT on macrophage production of angiogenic regulators.

Conclusion

PDT is a promising treatment against cancer that offers several advantages over the conventional oncology therapies, such as its high selectivity and low systemic toxicity. However, numerous survival strategies would arise in cancer cell granting an innate or acquired resistance to the treatment. A great among of proteins and molecular via are implicated in resistance to PDT, including MDR transporters, antioxidant enzymes, adhesion proteins and the extracellular matrix components, HSPs, IAPs andBcl-2 family proteins, explaining in this chapter. In addition to these survival strategies, an extensive list of other molecules isbeen studied, such as the proteins of the extrinsic apoptotic via, transcription factorse.g.NF-κB(nuclear factor kappa B), the apoptotic protein p53 and the immune system of the patient.

The complexity of the resistance phenomenon is associated with the complexity of the mechanisms triggered by PDT. Elucidation of the mechanisms of PDT action and its relation with the resistance will provide with a more effective treatment and a better future for oncologic patients.

PDT is still considered to be a new and promising antitumor strategy. Its full potential has yet to be shown, and its range of applications alone or in combination with other approved or experimental therapeutic approaches is definitely not exhausted. This chapter establishes a wide range of possibilities by which PDT could be optimized.

PDT is both a fascinating and a controversial area because it combines many drugs, protocols, light sources. Therefore, future research should be more complex and should include analysis of multiple contributing factors and outcomes of the treatment. Thus, the understanding of basic principles as well as the resistant mechanisms, the applicant in angiogenesis and the knowledge about agents and action mechanisms becomes imperative for the appropriate use of the techniques towards the best results possible against cancer disease.

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Chapter 9

Photodynamic Therapy in Dermatology: What's New

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Abstract

In dermatology, topical photodynamic therapy (PDT) is a well established treatment modality which has mainly shown to be effective for dermato-oncologic conditions like actinic keratosis, Bowen's disease, in-situ squamous cell carcinoma and superficial basal cell carcinoma. However, a therapeutical benefit of PDT is also evident for inflammatory dermatoses like localized scleroderma, acne vulgaris and granuloma annulare as well as for aesthetic indications like photo aged skin or sebaceous gland hyperplasia. Recent work has been focused on the development and evaluation of topical photosensitizers like the hem precursor 5-aminolevulinic acid or its methyl ester inducing photosensitizing porphyrins. These drugs do not induce strong generalized cutaneous photosensitization like the systemically applied porphyrins or their derivatives. For dermatological purposes incoherent lamps or LED arrays can be used for light activation. Depending on the applied light dose and the concentration of the photosensitizer either cytotoxic effects resulting in tumor destruction or immunomodulatory effects improving the inflammatory conditions occur.

More indications are emerging for the use of PDT in Dermatology that shall be discussed and highlighted in the following chapter.

History

The very first attempts to apply PDT to treatment of tumors and other skin diseases, such as lupus of the skin and chondylomata of the female genitalia, were performed by the group of von Tappeiner in 1903-1905. They tried a number of dyes: eosin, fluorescein, sodium

dichloroanthracene disulfonate and ‘Grubler’s Magdalene red’. The dyes were in most cases topically applied, but intratumoral injections were also attempted. Favorable results were reported, but there was no long-term followup, and PDT was soon forgotten, probably because of the advent of ionizing radiation in cancer therapy. The story of how this German group hit upon the idea of using dyes as biological sensitizers for light is fascinating: one of von Tappeiner’s students, Oscar Raab, was investigating the toxic effects of acridine on paramecia. In one experiment the paramecia survived incubation with a given acridine concentration for about 1.5 h, while in another experiment they survived for about 15 h under identical conditions, except, as recorded by the observant student, during one of the experiments there was a heavy thunderstorm. So he started to wonder whether light might have played a role-this resulted in the discovery of photodynamic action. The group performed a large amount of work on photosensitization, discovered that oxygen was required for the photodynamic effect and summarized their work in a book 1201. [1].

New Photosensitizer

Methelene Blue

Methylene blue (MB) is a molecule that has been playing important roles in microbiology and pharmacology for some time. It has been widely used to stain living organisms, to treat methemoglobinemia, and lately it has been considered as a drug for photodynamic therapy (PDT). In this review, fundamental photophysical, photochemical and photobiological characteristics of this molecule and evolved to show in vitro and in vivo applications related to PDT. The clinical cases shown include treatments of acne, psoriasis, basal cell carcinoma, Kaposi’s Sarcoma, melanoma, virus and fungal infections. MB has the potential to treat a variety of cancerous and non-cancerous diseases, with low toxicity and no side effects.

Photophysics

Methylene blue is a widely known histological dye that has been in use for many years. [2] It belongs to the phenothiazinium class of compounds. The characteristic color of MB is caused by the strong absorption band in the 550—700nm region with maximum molar absorptivity of 85,000M−1 cm−1 at 664nm. MB absorption spectrum is concentration-dependent due to dimerization, whose equilibrium constant is 3.8×103 M−1 in water. [3]

Photochemistry

MB monomers and dimers have distinct absorbance spectra. Monomers have maximum at 664nm and dimers at 590 nm. The difference in absorption between monomers and dimers facilitate the calculation of each species concentrations present in solution. Note that in 20 _M aqueous solution only MB monomers are present. However, after in vivo local injection of 2% MB solution in a basal cell epidermal tumor at the arm of a patient, maximum

absorption at 580nm was observed, indicating the presence of MB dimers in the tumor tissue. As it will be mentioned below, monomers and dimers may be involved in different kinds of photochemical reactions, which may affect the mechanism and efficiency of cell kill. [3]

New Source of Light

An inexpensive light source was developed called RL50® that emits light that covers the red spectrum region from 600 to 750 nm. The RL50® spectral radiance also overlaps with the absorbance spectrum of the MB solutions. [4]

Eosin

Eosin is a fluorescent red dye resulting from the action of bromine on fluorescein and used in lipstick manufacture and it is FDA approved [5] Von Tappeimer and Jesionek have been mentioned to be the pioneers in the field of PDT and in 1904; they used topical Eosin (5%) and light to treat skin cancer, lupus vulgaris, and condylomata. [6] Eosin acted as potent photosensitizer inside cells when activated by light in the presence of oxygen. Twenty patientsof primary axillary hyperhidrosis were treated using intense pulsed light with a 400 nm filter, 20 ms pulse duration and 25 J/cm² fluence, once weekly, for 4 sessions after applying Eosin liposomal hydro gel to the right axilla for 1 hour and placebo gel to the left. Efficacy was measured by hyperhidrosis disease severity scale (HDSS), and assessment of sweating area reduction using the minor iodine starch test. A 90.1 % reduction in the area of hyperhidrosis of the right axilla was obtained versus 2.2% reduction in the placebo site. A 3-point improvement on the 4-point (HDSS) was reported in 7 patients (35%), a 2-point improvement was reported in 9 patients (45%) and 1- point improvement was elicited in 4 patients (20%). Three patients (15%) reported a 1-point improvement from HDSS score 4 to score 3 at the placebo site. No recurrence occurred during the 8 month follow-up period. [7]

New Delivery System: ‘Nano’ Strategies for Photosensitizer

Photodynamic therapy (PDT) has developed over last century and is now becoming a more widely used medical tool having gained regulatory approval for the treatment of various diseases such as cancer and macular degeneration. It is a two-step technique in which the delivery of a photosensitizing drug is followed by the irradiation of light. Activated photosensitizers transfer energy to molecular oxygen which results in the generation of reactive oxygen species which in turn cause cells apoptosis or necrosis. Although this modality has significantly improved the quality of life and survival time for many cancer patients it still offers significant potential for further improvement. In addition to the development of new PDT drugs, the use of nanosized carriers for photosensitizers is a promising approach which might improve the efficiency of photodynamic activity and which can overcome many side effects associated with classic photodynamic therapy The general

advances made in nanomedicine and progress in the use of photosensitizers for PDT make the development of photoactive nanoparticles an obvious choice for current research; in addition it is considered a ‘‘hot topic’’ by funding agencies. This applies both to the development of anti-cancer drugs and of sensors for tumor indication and imaging [52] or for other applications of PDT such as neovascular disorders (e.g., age-related macular degeneration proliferative diabetic retinopathy, corneal angiogenesis). There are many ways to modify photosensitizers to improve effect of photodynamic therapy. PS can be modified by encapsulated them in delivery agents such as liposomes, micelles, ceramic based nanoparticles gold nanoparticles, and polymer nanoparticles Liposomes, for example, are able to encapsulate hydrophobic as well as hydrophilic drugs. Liposomal formulations show the ability to decrease the tendency of photosensitizer to aggregate and improve the tumor-selective accumulation. Micelles resist elimination by the RES which increases their circulation in the body and ability to deliver drug to the target cells. They also can encapsulate pharmaceuticals poorly soluble in water and are very biocompatible [38]. Biodegradable and non biodegradable nanoparticles encapsulated photosensitive drugs have a variety of advantages. Due to their small sizes they are not removed from the body by the RES system which leads to longer half-life times. They have a strong ability to protect encapsulated agents, are compatible with biological systems, and their surface can easily be modified with functional groups such as antibodies or other ligands to improve selectivity. Another possibility is to use nanodiamonds (ND). They are biocompatible, have minimal cytotoxicity and are commercially available carbon nanomaterials with a diamond structure at a nanometer scale and offer potential for medical applications. They are suitable for controlled drug delivery due to their capability to slowly and consistently release drugs, have a precise particle distribution, a high surface area to volume ratio and a substantial capacity for drug loading. Moreover, NDs are stable in water which makes them a promising and important tool, provided their retention time is not too long. Photosensitizers can also be modified using dendrimers, highly complex molecules with a core, branches and end.

Light Sources

Both laser and non-laser light sources are available for PDT. [8] The older lasers, including the argon laser, the neodymium: yttrium–aluminum–garnet (Nd:YAG) laser and the gold vapor laser, are now being superseded by more compact and less expensive solid state diode lasers. Non-laser lights, including filtered halogen or xenon arc lamps, blue light fluorescent tubes and light emitting diode (LED) arrays are useful for treating large areas of skin.

Non-Coherent Light Sources

Advantages

- Due to large illumination fields, useful in the treatment of large skin lesions
- Low cost and easy availability
- Different photosensitizers with varying absorption maxima can be used

Lasers in PDT

Combinations of light and chemicals are widely used to treat skin diseases. The concept of PDT was introduced for the main branch of this use. PDT is a promising modality for management of tumors and non-malignant skin diseases. It is based on the administration of a photosensitizer that selectively localizes in the target tissue. Exposure of the lesion to visible light in the presence of oxygen results in photodamage and subsequent tissue destruction. [9] Shortly after the invention of lasers in 1960, they were brought into medical use. Fundamental features, such as coherence and monochromaticity of laser light, made them excellent tools for a number of applications: in surgery, treatment of hemangiomas, skin rejuvenation, hair removal, etc.. [10]

Since coherence is lost within a few tenths of a millimeter of penetration into human tissue, this property is not necessary for PDT. Non-coherent light is, therefore, frequently used for irradiation of neoplasms. Non-coherent light sources differ fundamentally from lasers in output characteristics. Lasers and non-coherent light sources have been used for PDT and usually show similar efficacies. [11] Non-coherent light sources are relatively inexpensive, stable and easy to operate, and require little maintenance. Noncoherent, filtered, light sources often emit light with a larger bandwidth than that of lasers and LEDs. A comparison of two such light sources is, therefore, not straightforward. Careful dosimetric considerations are required. [12]

Photodynamic therapy using a pulsed laser is becoming popular, but its cytotoxic effect is still not clear. In the case of PDT using a continuous wave (CW) light, oxygen consumption may become a key factor in the determination of PDT effects. Many researchers have shown that oxygen depletion by PDT using CW light is substantially changed by fluence rate. CW light with a high fluence rate causes significant oxygen depletion, resulting in reduction of PDT effects. [13] On the other hand, enhanced PDT effects have been demonstrated when either CW light with a lower fluence rate or fractionated light is used. In addition, oxygen level in the cells exposed to CW light significantly affects the degree of photobleaching of a photosensitizer. [14]

The suggested mechanism of PDT using pulsed light is basically similar to that using CW laser, dependent on the present light conditions. This is because, regardless of laser source, the cytotoxic effect had a direct relationship to both the oxygen consumption during PDT and the resultant photobleaching after PDT. Thus, common mechanisms are suspected; however, cytotoxic efficiency appeared to be different, depending on the laser source. [15]

Another plausible reason for the difference between cytotoxic efficiency in PDT using a pulsed laser and a CW laser has been reported. Miyamoto et al. have shown that PDT using pulsed light induces a particular type of cell death, which is different from that induced by PDT using CW light. [16]

Nevertheless, further investigation is required to elucidate the mechanistic details of the decreased effect of PDT using a pulsed laser. Commonly used lasers for PDT are pulsed dye laser and diode lasers. In a study assessing the safety and efficacy of the long-pulsed pulsed dye laser (LP PDL) (595 nm) with PDT for the treatment of actinic cheilitis (AC), 21 patients were treated with a 20% ALA solution followed by activation with LP PDL. [17] Of these, the condition in 37% cleared with a single treatment, 68% cleared after 1.8 treatments, and 21% cleared after three treatments. In three patients with erosive AC, postoperative impetiginization occurred.

Two pulsed light sources available for the treatment of some aspects of cutaneous photodamage are the flashlamppumped pulsed dye laser and the filtered flashlamp/intense pulsed light (IPL). These have recently been used in conjunction with ALA for photodamage. Strasswimmer and Grande [18] demonstrated that IPL and PDL had a faint dose–response effect on PDT activation but were less potent than a smaller fluence of CW blue light.

Karrer et al. [19] studied the efficacy of PDT with ALA using a long-pulse (1.5 ms) tunable flashlamp-pumped pulsed dye laser (LPDL) in vitro and in vivo. HaCaT human keratinocytes were incubated with ALA and irradiated with LPDL at 585 nm, 595 nm and 600 nm compared with an incoherent light source (580–740 nm). Also, 24 patients were treated topically with 20% ALA PDT emulsion irradiated by either an incoherent light source or 585 nm LPDL. Maximal cytotoxic effects in vitro were achieved with the LPDL at 585 nm or the incoherent Complete remission was achieved in 79% of 100 patients with actinic keratosis (AK) treated by ALA and LPDL and in 84% of 100 patients with AK treated by ALA and the incoherent lamp. Pain during light treatment was significantly reduced by with the LPDL. Control lesions (LPDL without ALA) did not clear

Advantages of Using Lasers as a Light Source for PDT

Maximum effectiveness can be achieved if the wavelength of the laser matches the peak absorption of the photosensitizer, due to the monochromatic quality possessed by the lasers.

High irradiance produced by lasers helps minimize therapeutic exposure time.

Lasers can be delivered to internal organs such as gastrointestinal tract and lungs, when conjugated with fiberoptics.

Disadvantages

- Expensive modality
- High maintenance
- Coupling with fiberoptics renders lasers useful only to small lesions on the skin

The choice of light source may be influenced by the intended applications. For dermatologic purposes, where the light has to penetrate the skin, the scatter is high and so the effective depth of ALA PDT is 1–3 mm at 630 nm. Number and size of lesions, need for a portable compact source with a smaller field source, flexibility, treatment times and cost are the other important factors taken into consideration in the choice of a light source

Therapeutic Indications of PDT

Regarding oncologic indications, AK, nodular or superficial BCC and – since 2006 – Bowen's disease are approved indications for MAL. Approval for ALA was given by the FDA for the combination with blue light in the treatment of AK and most recently (2009) the ALA patch was approved in combination with red light for the treatment of AK in Europe.

Actinic Keratosis

AK evolve in UV-exposed areas and are the most frequent precancerous lesion of the skin. They often impose in a widespread pattern, often necessitating field cancerization. The treatment of AK is mandatory as they have the potential to develop into invasive SCC. In addition to PDT, there exist a number of registered chemical and/or immunological treatment options for the treatment of AK 5-fluorouracil (5-FU, Podophyllin, Imiquimod, Diclofenac, PDT). [20] The choice of the suitable treatment depends on numerous factors, one of which is the number and localization of the lesions that have to be treated. For therapy of single lesions, surgical or physical treatment options like cryotherapy, laser ablation or surgery might be favorable. In contrast, PDT may be the first choice for the therapy of multiple lesions, in particular for AK on the scalp and face or in cases of basal cell nevus syndrome. [21] A great advantage of PDT as compared with chemical and/or immunological treatment options is the fact that the patient himself does not have to take care of the treatment over weeks, which is a great compliance-related problem.

Basal Cell Carcinoma

Various studies concerning ALA/MAL-PDT for BCC have been performed over the years. [22] The weighted average complete clearance rates calculated from 12 studies (follow-up periods: 3–36 months) were 87% for superficial BCC (*n*=826) and 53% for nodular BCC (*n*=208). Available compiled data from other trials have shown an average of 87% for superficial BCC, and 71% for nodular BCC. [24] In order to ameliorate poor outcome after PDT of thicker BCC lesions, Thissen *et al*. [23] treated nodular BCC (23 patients, 24 lesions) once with ALA-PDT (incoherent red light; 100 mW/cm^2, 120 J/cm^2) 3 weeks after debulking of the BCC. The former tumor areas were excised 3 months later and histopathologically evaluated for residual tumor. Twenty-two (92%) of the lesions showed complete remission, both clinically and histologically.

However, even if all clinical studies qualify PDT as an effective treatment of BCC, Moh's micrographic surgery shows generally higher cure rates as compared with PDT. Besides, the relatively short follow-up of most of the performed studies has to be considered. Mandatory indications for surgical treatment are histological subtypes like pigmented or morpheic BCC, BCC located in the area of the facial embryonic fusion clefts as well as all BCC thicker than 3 mm if no debulking procedure is performed before PDT.

Bowen's Disease and Initial SCC

Bowen's disease is approved for MAL-PDT since 2006 and is – as a planar epithelial precancerous lesion – highly suitable for PDT. In a recent study by Salim *et al*. [24] ALA-PDT was compared with topical 5-FU in the treatment of Bowen's disease. In this bi-center, randomized, phase III trial, 40 patients with one to three lesions of histologically proven Bowen's disease untreated previously (40 patients, 66 lesions) received either PDT (*n*=33) or 5-FU (*n*=33). ALA 20% in an oil/water-emulsion was applied 4 h before illumination with an incoherent light source (Paterson lamp, Phototherapeutics, λ_{em}=630±15 nm; 50–90 mW/cm^2,

100 J/cm^2). Treatment with 5-FU was once daily in week 1 and then twice daily during weeks 2–4. At the first follow-up at week 6, both ALA-PDT and 5-FU application were repeated, if required. Twenty-nine of 33 lesions (88%) treated with PDT showed complete response, versus 67% after 5-FU (22 of 33). After 1 year of follow-up, further recurrences reduced the complete clinical clearance rates to 82% and 42%, respectively. [27]

Relatively New Non Oncologic Applications

Onychomycosis are becoming more prevalent especially in immune-suppressed patients and are of difficult treatment. Topical and oral medications are possible. The treatments are long and the oral medications are hepatotoxic with less than 80% complete response. PDT has the potential to become new treatment approach with smaller toxicity. [25]

More than 60 patients were treated by Tardivo JP et al [4] using this protocol with complete response ; drip 2% MB dissolved in acetone between nail plate and nail bed; apply light irradiation with RL 50 with final dose of 18 J/cm2; and take pictures and repeat the protocol after 30 days if necessary.

Acne

Liposomes loaded methylene blue (LMB) was prepared and studied for different pharmaceutical properties and formulated in hydrogel (MB 0.1 %). Permeability and selective sebaceous gland targeting in mice skin was studied. Gel containing LMB was used for treating 13 patients complaining of mild-moderate acne vulgaris once a week for two weeks. Efficacy evaluation included changes in lesions counts, clinical assessments of clinical improvement by patients and evaluating dermatologists. Pain and local adverse effects were also evaluated.

After only two sessions, there was a 83.3% reduction in the number of inflammatory acne lesions and a 63.6% reduction in the number of non-inflammatory acne lesions. At 12 weeks, 90% of patients had a moderate-to-marked improvement of their acne in the treated areas. Most patients had no pain; also no serious adverse side effects were recorded. Slight transient hyperpigmentation was seen only in three patients. [26]

Psoriasis

Topical treatment of resistant psoriatic plaque stage lesions may be difficult and the systemic therapies seem inappropriate. The efficiency of the photodynamic therapy (PDT) of MB photo-activated using 565 mW Light emitting diode (LED) 670 nm was evaluated in patients with resistant plaque psoriasis. The patients were subjected to 2 sessions weekly for a maximum of 12 sessions, skin biopsies from each patient in the beginning and at the end of the sessions were taken for histopathological studies. Results showed that the sixteen patients experienced complete clearance of their treated lesions. Skin appeared normal in color, texture, and pliability with no complications indicating the lack of skin sensitivity.

Histopathological examinations showed nearly normal epidermis at the end of all sessions. The authors concluded that the prepared hydrogel was safe, stable, and very effective. The results are encouraging to accept MB as a photosensitizer for PDT and as a safe and effective method for treatment of selected cases of resistant localized psoriasis. [27]

Leishmaniasis

A recently published placebo-controlled randomized study [28] compared the effectiveness of PDT to topically applied Paromomycin in patients (n=60) with cutaneous leishmaniasis. The patients were randomly assigned to three treatment groups (n=20 each). Group 1 was treated with weekly topical PDT. Groups 2 and 3 received twice-daily topical paromomycin and placebo, respectively. The duration of treatment was 1 month for all groups.

Patients were followed-up for 2 months after the end of treatment. The authors reported that 57 patients with 95 lesions completed the study. At the end of the study, complete improvement was seen in 29 of 31 (93.5%), 14 of 34 (41.2%) and four of 30 lesions (13.3%) in groups 1, 2 and 3, respectively.

At the same time point, 100%, 64.7% and 20% of the lesions had parasitological cure in groups 1, 2 and 3, respectively. The authors concluded that topical PDT can be used safely as a rapid and highly effective treatment alternative for cutaneous leishmaniasis. [28] Enk *et al*. [29] performed ALA-PDT twice on 11 patients with 32 cutaneous leishmaniasis lesions and reported a lack of any parasites in 31 out of 32 lesions and the average reduction in size was 67% following ALA-PDT. Cosmetic results were excellent and there was no recurrence within 6 months. Further case reports document the successful use of PDT in cutaneous leishmaniasis. [30, 31] Regarding the mechanism of action it is proposed that PpIX leads to the generation of singlet oxygen, which induces the destruction of the parasite's coat, oxidates bacterial lipids and proteins. [32]

Antifungal Applications

Fungal infections have been increasing in incidence during the last 20 years and represent a significant health burden as a major pathogen in critically ill patients. [33] *Candida* currently represent the third leading cause of bloodstream infections in the U.S., and disseminated candidiasis has an associated mortality rate >25%. It has been suggested that the increase in these infections might be due to increasing use of immunosuppressive drugs, antibiotics, prosthetic devices, and surgeries. [33] Compared with the myriad of antibiotics that exist, antifungal treatment options are limited, and to make matters worse, drug resistance to antifungal agents might be increasing. [34] Hence, there is a critical need for alternative antifungal treatments. Although there is growing interest in exploring the clinical application of PDT as an antifungal therapy, little has been published on this topic aside from preliminary in vitro studies.

Smijs and Schuitmaker [35] published the first study that demonstrated the susceptibility of dermatophytes to PDT by using various photosensitizers. The study found that phthalocyanines and Photofrin had fungistatic effects, whereas porphyrins had a fungicidal

effect on *Trichophyton rubrum*. The authors recommended PDT as a promising entity, which should be further investigated as a treatment for tinea infections. Since then, several in vitro findings have demonstrated that dermatophytes and yeasts can be effectively targeted by PDT.

In a study conducted by our laboratory at Case Western, Lam et al [36] showed that PDT can effectively induce apoptosis of *Candida albicans* grown in culture, indicating that this modality could feasibly be developed as a treatment option for candidiasis.

With the lack of data from clinical trials, it is uncertain at this point what PDT's place will be in the treatment of mycoses. So far, studies indicate that the initial response to PDT is high; however, the rapid recurrence makes PDT an unacceptable treatment option. Further investigations are warranted to look for protocols that can reduce the rate of recurrent disease, because PDT can be a safe and selective method of treatment that would be advantageous where risk of drug-resistant fungal strains is a serious concern.

Conclusion

There is abundant evidence in literature demonstrating that PDT is effective for the treatment of nonmelanoma skin cancers as well as non-oncological cutaneous conditions. Topical PDT in dermatology is approved for the treatment of AKs in the U.S., and studies suggest that it can also be recommended as a first-line treatment for Bowen disease and superficial BCC.

Because PDT is relatively noninvasive and capable of field treatment, it might be the preferred mode of treatment in patients who are poor surgical candidates or those who have multiple or cosmetically sensitive lesions. PDT is associated with faster recovery periods and has consistently demonstrated superior cosmetic outcome over conventional treatments. In respect to the nonmalignant conditions, currently available evidence supports that PDT can be a safe option for the treatment of acne, psoriasis, warts, and certain cutaneous infections. However, larger clinical trials are needed to evaluate its effectiveness, especially in comparison with existing treatments.

In addition, there is currently a lack of consensus regarding skin preparation, incubation time, the choice of light source, and duration of light exposure. Hence, future advances in the application of PDT for these various conditions should involve the development of standardized treatment protocols. Overall, PDT is a generally well-tolerated treatment modality for a wide range of malignant, inflammatory, and infectious processes, and its use in dermatology is expected to increase in the future.

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Chapter 10

Dendrimer Based Drug Delivery System for Efficient Delivery of Hydrophilic Photosensitizer in Photodynamic Therapy of Cancer

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Abstract

Photodynamic therapy (PDT) is a promising treatment methodology whereby diseased cells and tissues are destroyed by reactive oxygen species (ROS) by using a combination of light and photosensitizers (PS). The major problems of most of the photosensitizers to limit their usage are due to their intrinsic toxicity and insufficient lipophilicity. In this chapter, we evaluate the potential of polyamidoamine (PAMAM) dendrimers for the delivery of model photosensitizer (Rose Bengal, RB) and evaluation of its phototoxic efficiency towards a model cancer cell line. The spherical, nanoscaled dendrimers could efficiently encapsulate RB and showed characteristic spectral responses.

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The controlled release kinetics of RB from dendrimer–RB formulation was clearly evident from the in vitro drug release study. ROS generation was confirmed in dendrimer–RB system upon white light illumination. Photosensitization of Dalton's Lymphoma Ascite (DLA) cells incubated with dendritic formulation caused remarkable photocytotoxicity. Importantly, the use of dendritic drug delivery system reduced the dark toxicity of RB which makes them as potential drug delivery vehicle for photodynamic therapy.

Keywords: Photodynamic therapy, Dendrimers, Rose Bengal, Phototoxicity, Reactive Oxygen Species

1. Introduction

Photodynamic therapy (PDT) is a method of clinical treatment whereby diseased cells and tissues are destroyed by a combination of light and special drugs called photosensitizers (PS) (Lopez et al. 2010; Silva et al. 2009; Roy et al. 2003). In addition, the presence of adequate molecular oxygen in the tissue is also required (Krishnamoorthy et al. 2011). Mechanistically, it has three phases viz. (i) excitation of drug molecules, (ii) generation of toxic oxygen and (iii) cell death. These components, tolerated singly by the diseased cells, generate cytotoxic oxygen-based molecular species when combined in proper dosage and concentration (Roberson et al. 2009; Guo et al. 2010). PDT is non-invasive and is recognized as a useful initial treatment for malignant tumors (Dolmans et al. 2003; Macdonald et al. 2001). PDT using porfimer sodium (Photofrin®) has been approved for the treatment of oesophageal cancer in the United States and Canada, early and late stage lung cancer in the Netherlands, bladder cancer in Canada, and early stage lung, oesophageal, gastric and cervical cancers in Japan (Fisher et al. 1996).

In general, photosensitizers can be divided into two types viz. (i) hydrophilic and (ii) hydrophobic compounds. The major drawbacks of the hydrophobic photosensitizers are that they cannot be simply injected intravenously since they form aggregates in solution that restricts their medical applications (Orenstein et al. 1996; Lobouebe et al 2006). Hence hydrophobic photosensitizers need complex formulation for systematic delivery (Fenga et al. 2004; Shive et al 1997). Hydrophilic photosensitizers are advantageous than hydrophobic photosensitizers since they can be easily delivered intravenously and significantly improve tumor killing (Moore et al. 2009; Vrouenraets et al 2002). However, hydrophilic photosensitizers poorly accumulate in tumour cells as it finds difficulty in crossing cell membranes. This is mainly because the cellular transport systems in cancer cells are slowly accelerated for hydrophilic drugs to pass through when compared to normal cells (Kessel et al. 1981).

Rose Bengal (RB) is a hydrophilic photosensitizer with a high absorption coefficient in the visible region of the spectrum at 552 nm showing good quantum yield of singlet oxygen (Kochevar et al. 1996). Although it has potential in photodynamic therapy of tumors, its tendency to aggregate in solution under physiological condition decreases the yield of reactive oxygen species (ROS) (Killig et al. 2004). Therefore, it is essential to have an appropriate formulation for the delivery of this hydrophilic photosensitizer in therapeutic levels. The ideal drug delivery system for carrying PDT should be biodegradable, have

minimum toxicity, incorporate the photosensitizer without loss or alteration of the sensitizer activity and provide an environment where the photosensitizer can be administered in monomeric form (Konan et al. 2002). Importantly, the delivery system should enable selective accumulation of the PS within the diseased tissue in therapeutic concentrations with little or no uptake by non-target cells (Chatterjee et al. 2008).

It is expected that charged or slightly lipophilic nanoscaled drug delivery systems can be used for efficient delivery of highly hydrophilic photosensitizers for PDT of cancer. In this regard, the use of dendrimer-based nanocarriers is a promising method for the tumor specific delivery of PS.

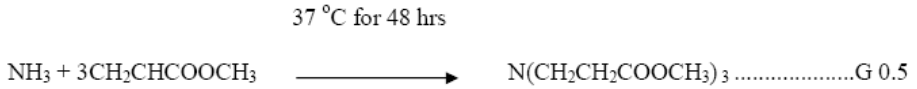
Nanoparticles, such as nanospheres and nanocapsules possess high impact in delivery system as PS carriers because they can meet all the requirements for an ideal PDT agent (Premanathan et al. 2011; Murday et al 2009; Koo et al. 2005). Dendrimers can be considered as the most versatile, compositionally and structurally controlled synthetic nanoscale building blocks available today (Koda et al. 2008; Bechet et al. 2008). Dendrimers have high degree of molecular uniformity, loading capacity, biocompatibility and a highly- functionalized terminal surface that facilitates the modification of the solubility of drugs to help target the drug to its therapeutic sites, or to alter the release profile of the therapeutic agent (Svenson et al. 2005; Jansen et al. 2008). With the aim of improving the drug delivery and release kinetics suitable for carrying PDT and diminishing the dark toxicity of RB, dendrimer based delivery system could be a better choice.

The present study relies on polyamidoamine (PAMAM) dendrimers as an efficient drug delivery system for a well known hydrophilic photosensitizer, Rose Bengal and was evaluated by investigating the interaction between the dendrimer and RB and the photodynamic efficacy. However our studies explored the influence of G2.5 PAMAM+RB on Dalton's Lymphoma Ascite (DLA) cancer cell lines. A potential application of PAMAM dendrimers as an efficient drug delivery system for a hydrophilic photosensitizer will provide new opportunities in nanomedicine for PDT of cancer.

2. Results and Discussion

2.1. Synthesis of PAMAM Dendrimer

PAMAM dendrimers with various generations have been synthesized using a divergent method. The synthesis of PAMAM dendrimers undergoes Michael addition reaction (Hedden et al. 2003). Ammonia and 100 equiv. of methylacrylate (MA) were dissolved in methanol, respectively. Ammonia solution was added to MA solution drop wise. Reaction mixture was stirred in 37 ^{o}C for 2 days. After reaction, solvent was removed by rotary evaporator and residual product was stored in vacuum.



PAMAM G0.5 and 100 equiv. of ethylenediamine (EDA) was dissolved in methanol, respectively. PAMAM solution was added to EDA solution and stirred at 50 °C for two days.

After reaction was completed, solvent was removed using rotary evaporator. Residual product was precipitated in ethyl ether by two times and stored in vacuum.

$$N(CH_2CH_2COOCH_3)_3 + 3NH_2CH_2CH_2NH_2 \xrightarrow{50\ ^{\circ}C \text{ for } 48 \text{ hrs}} N(CH_2CH_2CONHCH_2CH_2NH_2)_3 + 3CH_3OH \quad \text{.....G1.0}$$

The first generation dendrimer G1.0 was purified by three times centrifugation and redispersed in methanol. Addition of MA in proper molar ratios with G1.0 under heating (50°C) for 48 h results in production of G1.5 PAMAM dendrimer. Subsequent addition of EDA generates G2.0 dendrimer. Likewise, the chain reaction is continued till the synthesis for G2.5 dendrimer. The dendrimer was purified at each step by centrifugation for 30 min at 16000 g and resuspending in methanol solution. Finally the dendrimer solution was dialyzed against methanol water (1:10) mixture for 24 h in order to remove any non-reacted chemical species.

2.2. Encapsulation of Rose Bengal into the PAMAM Dendritic Box

The G2.5 PAMAM dendrimer and RB were mixed in the ratio of 10:1 in a solution of 20 ml of methanol and 5 ml of water. The resulting solution was vigorously stirred at 500 rpm for 24 h using a magnetic stirrer. After 24 h, the solvent was removed under vacuum using a rotary evaporator. The final product obtained was purified by centrifugation at 16000 g for 30 min followed by dialysis and stored at 4 ^{o}C.

2.3. Characterization of PAMAM Dendrimers

The PAMAM dendrimers of various generations (G0.5, G1.0, G1.5, G2.0 and G2.5) are synthesized using the Michael addition method as described in the previous section. The PAMAM dendrimers with ammonia as the core molecule possess an ester group (R-COO-R) and amine (R-CO-NH_2) group as terminal surfaces in the successive half and full generation. The FTIR spectra of all the dendrimers are represented in Figure 1. The FTIR spectra of the half generation (G0.5/G1.5/G2.5) shows a characteristic -C=O stretching vibrations around 1729 cm^{-1} due to the presence of free ester (C=O) group in the end surface (Kolhe et al. 2003). In addition to this, a band at 1000 cm^{-1} to 1200 cm^{-1} is also appeared which is assigned to the -C-O stretching mode. The FTIR spectrum of the full generation dendrimers shows characteristic N-H stretching vibrations around 1620-1650 cm^{-1} because of the reaction of ester with amine makes it as an amine end.

From the zeta potential values of various generations, it is possible to evaluate the nature of the surface groups present in the dendrimers (Tomalia et al. 2007). The half generation PAMAM dendrimers are electrically neutral and show positive potential values. The full generation PAMAM dendrimers are electronegative and they possess negative potential values.

The zeta potential data also confirmed the presence of the charged/neutral terminals at the surfaces. The zeta potential values of all the PAMAM dendrimers of various generations is given in the Table 1.

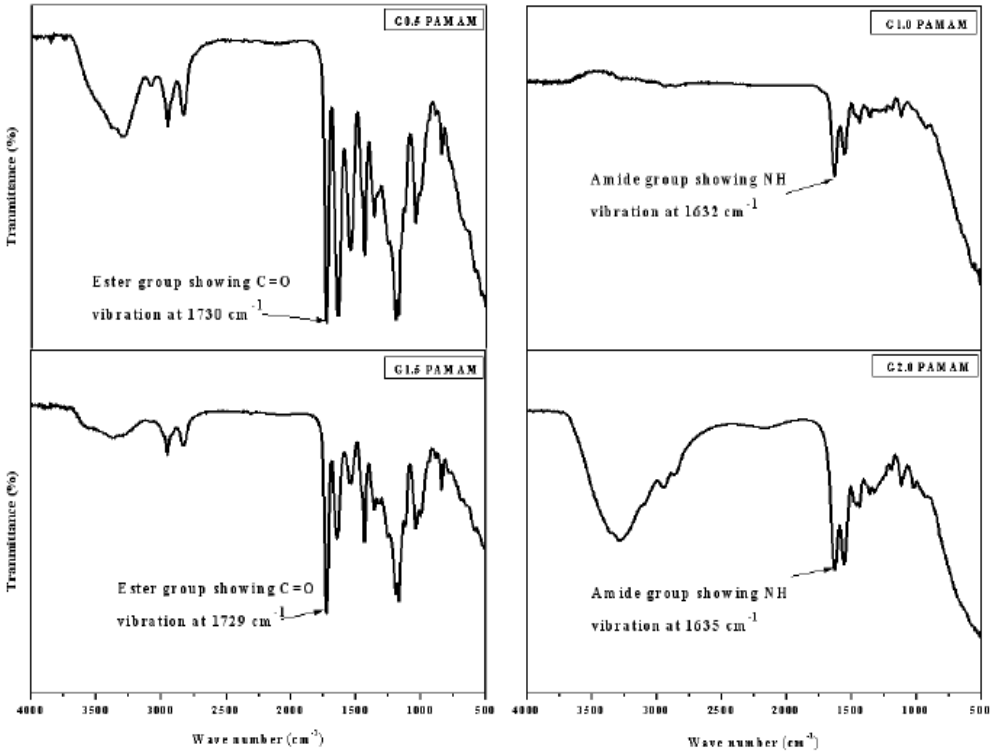


Figure 1. FTIR spectra of PAMAM dendrimers G0.5 to G2.0.

Table 1. Zeta potential values of PAMAM dendrimers

Generations of dendrimer	Zeta Potential (mV)
G0.5	1.82
G1.0	-0. 309
G1.5	1.69
G2.0	-0.543
G2.5	0.168

2.4. Dendrimer–Rose Bengal Interactions

The dendritic cavity present in the dendrimer molecules, enables them to hold the guest molecule (drug). Such host-guest interactions can be successfully followed spectrophotometrically. UV-visible absorption peak of G2.5 dendrimer and the RB drug in methanol is shown as Figure 2. The dendrimer shows an absorption band in the UV region around 270-280 nm and the free RB shows a peak at 552 nm (Fini et al. 2007). A weak band

appearing around 500 nm is a measure of aggregation of the RB molecules in solution (Xu et al. 1989). On mixing equimolar amounts of dendrimer and the drug, the band appeared in the visible region (around 552 nm) is shifted towards 545 nm. Because of the movement of drug molecules into the dendritic entity, non-covalent interactions between the RB and the internal cavities of PAMAM dendrimers makes such a blue shift (Cheng et al. 2007).

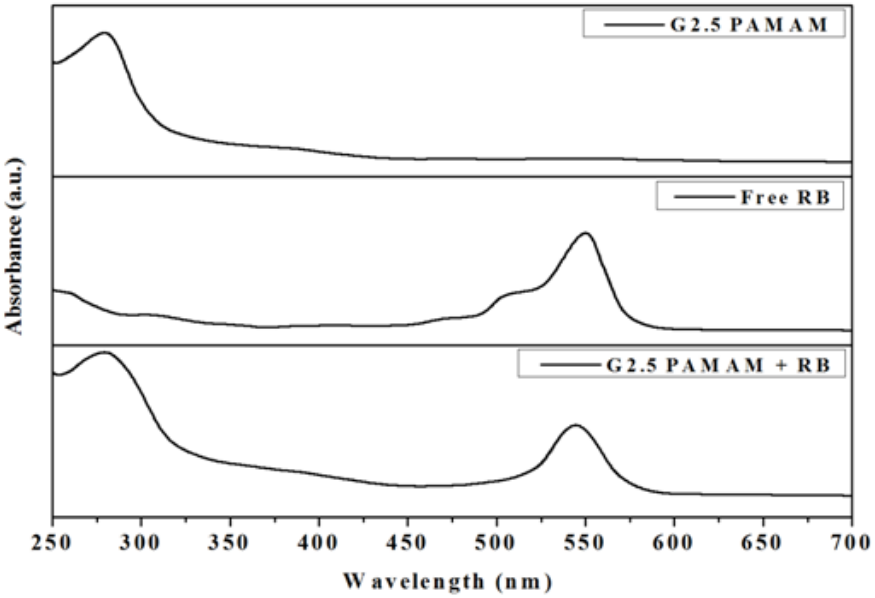


Figure 2. UV-vis spectra of G2.5 PAMAM dendrimers, free RB and G2.5 PAMAM+RB.

In addition to this, intermolecular interaction between the carboxyl group of RB and the terminal groups of PAMAM dendrimers also makes such blue shift (Gigimol et al. 2003). In the UV-vis. spectra of the G2.5 PAMAM+RB there is no observation of broad shoulder peak around 500 nm since on encapsulation, the delocalization of the lone pair of electrons present in the RB molecule with dendrimer (Finia et al. 2004). These results concluded that the RB molecules interact well with G2.5 PAMAM dendrimers through cavity encapsulation.

The host-guest interactions were further characterized by FTIR spectroscopy. The FTIR spectra of G2.5 PAMAM dendrimer, free RB and the G2.5 PAMAM+RB are shown in Figure 3. The FTIR of G2.5 PAMAM dendrimer shows a characteristic C=O stretching vibrations around 1729cm^{-1} which is assigned to an ester group (present as free terminal group in G2.5 dendrimer). The band at 1641 cm^{-1} is assigned to N-H deformation vibration present in the amide group (Kolhe et al. 2003). In addition to this, a 1000 cm^{-1} to 1200 cm^{-1} band is assigned to the C-O stretching mode. Peaks in the region 2800–3200 cm^{-1} corresponds to N–H stretching and C–H stretching vibrations (Devarakonda et al. 2007). The FTIR spectra of RB shows a characteristic C=O stretching at 1620 cm^{-1} (Jhonsi et al 2009). All the bands found individually on the dendrimer and the drug gets shifts if both are mixed together. The C=O and N-H deformation bands shifted to lower frequencies 1718 cm^{-1} and 1540 cm^{-1} which could be explained by intermolecular hydrogen bonding between electronegative atoms in the RB with the G2.5 PAMAM dendrimer. These observations reveal that the RB

molecules are encapsulated in the dendritic box through their carboxyl group via electrostatic interaction.

Zeta potential measurements enable the understanding of the interaction of RB and dendrimer moieties. G2.5 PAMAM dendrimer possesses a surface charge of +0.168 mV. Upon interaction with RB the surface charge of the dendrimer changed to -1.33 mV. This may be due to the presence of surface attached RB molecules that are having more electronegative chlorine atoms and a keto group in the molecule.

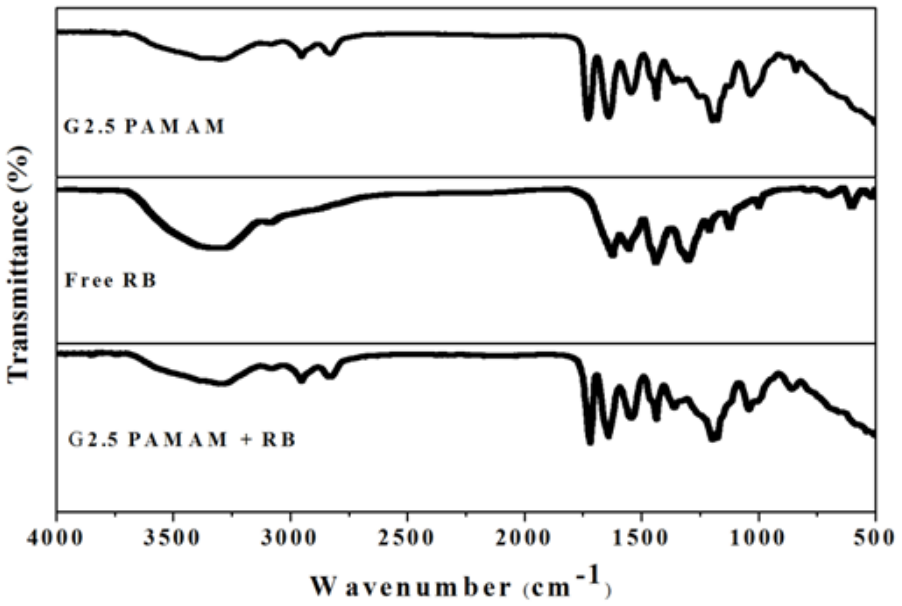


Figure 3. FTIR spectra of G2.5 PAMAM dendrimers, free RB and G2.5 PAMAM+RB.

Hence the above characterization techniques reveals that the RB molecules are encapsulated in the dendritic cavity and also some of the RB molecules are absorbed at the terminal surface of the PAMAM dendrimers.

2.5. Drug Loading and Encapsulation Efficiency of G2.5 PAMAM+RB Nanocapsules

High drug loading and better encapsulation efficiency is expected for an ideal drug delivery agent thereby reducing the quantity of the matrix materials for drug administration (Mohanraj et al 2006). The drug loading in the G2.5 PAMAM+RB is through the absorption technique. Hence the delivery system should be ideal in case of drug loading efficiency and drug release kinetics for carrying PDT, not suppressing the quantum yield of the PS after encapsulation. The amount of drug loaded into the dendrimer and the encapsulation efficiency of the G2.5 PAMAM dendrimers were measured spectrophotometrically during purification of G2.5 PAMAM+RB by centrifugation and are to be observed as 1.8 % and 92.5 % respectively.

2.6. *In Vitro* Drug Release Kinetics of G2.5 PAMAM+RB

In our G2.5 PAMAM+RB, the RB molecules are physically encapsulated in the cavity of the PAMAM dendrimer and a minimum quantity is absorbed at the terminal surface. The possible drug release kinetics is through diffusion process (Kedar et al 2010). Figure 4 shows the *in vitro* drug release profile of the RB encapsulated G2.5 PAMAM dendrimers followed spectrophoto-metrically for a period of 72 h.

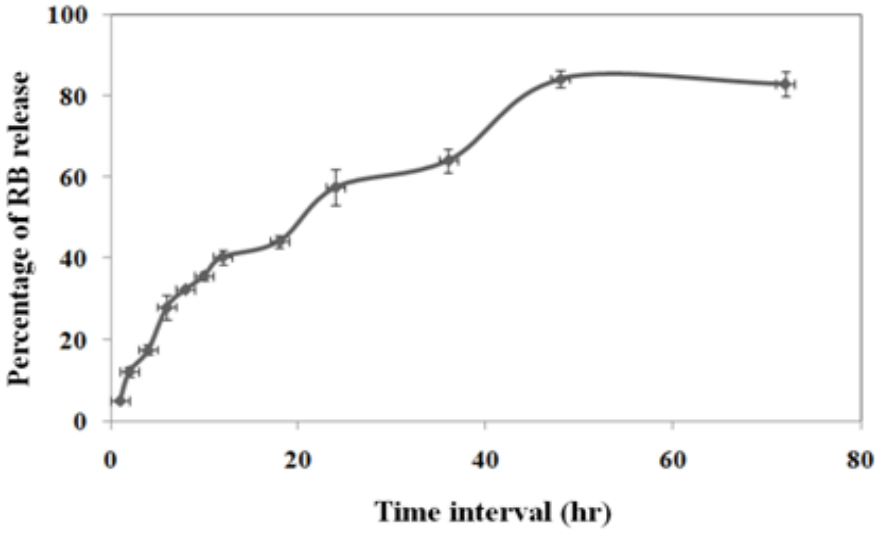


Figure 4. RB release kinetics from G2.5 PAMAM + RB.

It is observed that, the systematic release of RB after 12, 24 and 48 h are 35%, 50% and 74% respectively. After 72 h, 83% of drug release is noticed. From the graph, it is clear that, the rate of release of RB from the dendrimer at the initial stage is high whereas on the final stage it is found as low.

The quicker release in the initial hours is the release of small amount of RB attached onto the surface groups of the dendrimer. The drug release becomes somewhat slower i.e., after 12 h is probably due to the encapsulated drug which is present in the dendritic cavity or the inner core of PAMAM. Sustained release was noticed after 48 h. The drug release studies shows that G2.5 PAMAM dendritic system possesses excellent controlled release properties suitable for carrying PDT of hydrophilic photosensitizers.

2.7. ROS Quantum Efficiency of G2.5 PAMAM + RB

The significant factor influencing the PDT efficiency is the quantum yield of ROS generation from the PS. The ROS generation of free RB and its encapsulated form was evaluated by determining the quantum yield by iodide method. It is experimentally found that the $^1O_2$ quantum yield for free RB is 0.76. The $^1O_2$ quantum yield for G2.5 PAMAM+RB was measured as 0.71 from using the iodide method (Mosinger et al 1997). Figure 5 depicts the graphical representation of the change in absorbance of the iodide band (351 nm) against the

irradiation time for variously concentrated solutions of RB encapsulated in G2.5 PAMAM dendrimers.

It is clear from the Figure5. that the absorption of the iodide band increases with the increase in the concentration of the G2.5 PAMAM+RB, which indirectly show the increase in the ROS generation of G2.5 PAMAM+RB at higher concentration.

These results strongly demonstrate that the G2.5 PAMAM can be an ideal drug delivery agent since no such alteration in the ROS activity is noticed when compared to the activity of the free RB.

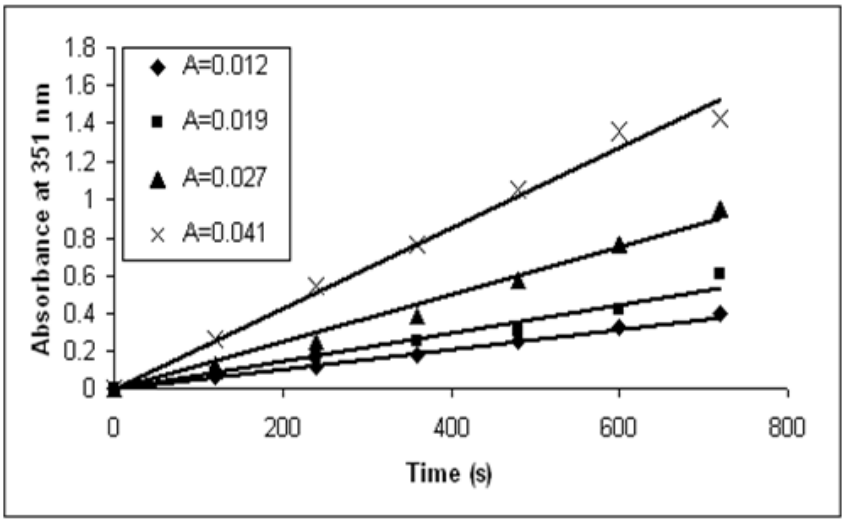


Figure 5. Estimation of quantum yield of ROS generation from the nanocapsules by the iodide method. Graph shows the change in absorbance of the iodide band (351 nm) against the irradiation time for variously concentrated solutions of RB encapsulated in G2.5 PAMAM dendrimers.

2.8. Phototoxicity and Dark Toxicity of G2.5 PAMAM + RB

The aim of this study was to investigate the photosensitizing activity of G2.5 PAMAM+RB against DLA cells and its efficiency as a potential PDT treatment for cancer. The cell viability of DLA cells upon photoirradiation as a function of concentration of G2.5 PAMAM+RB indicates the photodynamic effect *in vitro*. Figure 6 illustrates the photo-dynamic effect on the DLA cell line, as a function of the photosensitizer concentration. The free RB produced the lower photodynamic effect when compared to G2.5PAMAM + RB. The beneficial effect of the RB loaded dendrimers was mainly highlighted at a RB concentration of 510 nM. When the doses of the free RB and the G2.5 PAMAM+RB are increased to 510nM, it was found that the cell viability percentage for G2.5 PAMAM+RB treated cells was 24.9 % when compared to the 38 % cell viability for free RB treated cells. The phototoxicity results show that the G2.5 PAMAM+RB are more toxic to the DLA cells compared to the toxicity of free RB. PAMAM dendrimer is a hyper-branched molecule and the amount of drug loading is as low as 1.8% which ensures the uniform distribution of the photosensitizer in the dendritic matrix and this enables sustained drug release kinetics suitable for carrying PDT.

Low dark toxicity is one of the significant criteria for assessing the usefulness of photosensitizers, since the major side effects in clinical PDT result from the dark toxicity of photosensitizer to normal tissue. From Figure 7, at lower concentration of the free RB, the cell viability is 92% and an increase in concentration of the free RB to 510 nM makes it more toxic and the cell viability is reduced to 56 %. The dark toxicity of the RB loaded dendritic nanostructures exhibits non-toxic at lower concentrations and is less toxic at higher concentrations (the cell viability is 69.8% at 510 nM) as compared to the dark toxicity of free RB.

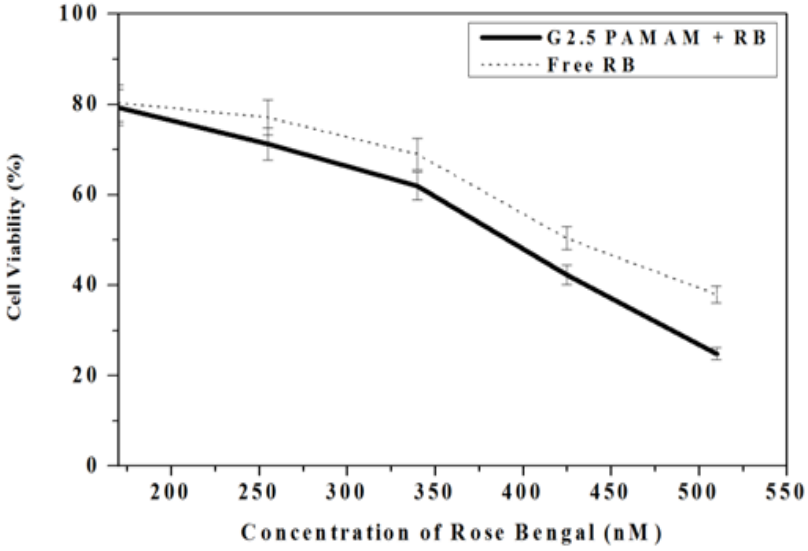


Figure 6. Phototoxicity of free RB and G2.5 PAMAM+RB.

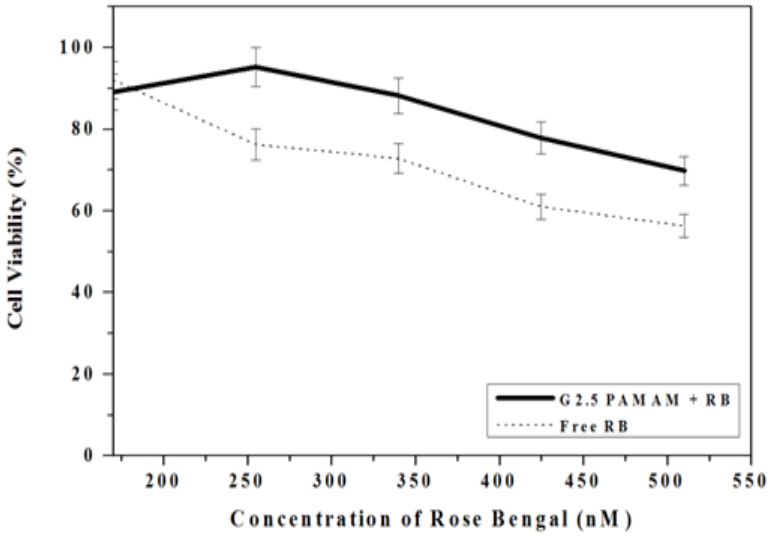


Figure 7. Dark toxicity of free RB and G2.5 PAMAM+RB.

The very low toxicity of G2.5 PAMAM+RB might result from the good biocompatibility and low toxicity of PAMAM dendrimers (Koda et al. 2008; Bechet et al. 2008; Svenson et al 2005). It can be highlighted that, differential toxicity was observed for G2.5 PAMAM+RB in the presence and absence of light against cancer cells. Therefore, the efficient photodynamic efficacy of G2.5 PAMAM+RB, together with the above mentioned advantages, makes this type of formulation for delivery of photosensitizer in PDT potentially very useful for clinical application.

Conclusion

In summary, PAMAM dendritic nanostructures could effectively deliver RB photosensitizers into cancer cells and produce enhanced photodynamic efficacy. The interaction between the PAMAM dendrimer and RB are investigated by UV-vis and FTIR spectra and zeta potential measurements.

Our systemic investigation of the dendrimer based formulation of RB shows that it is feasible to encapsulate even a hydrophilic photosensitizer with excellent encapsulation efficiency and release kinetics. Results showed that drug release was quite faster in the initial hours and above 80% was released in 72 h of time which was found to be more satisfactory for a water-soluble photosensitizer. It is observed that the PAMAM dendrimer based delivery of RB could retain its ROS generation property upon irradiation and also could reduce its toxicity by holding the RB molecules in the internal cavities.

Importantly, the dendritic formulation exhibited minimized dark toxicity within the concentration used and enhanced phototoxicity to DLA cells compared to the dark and phototoxicity of free RB. The key findings of our work with the above mentioned advantages ensure that PAMAM dendrimer based nanocarriers of PS delivery should be a promising candidate for PDT.

4. Material and Methods

4.1. Materials Used

Methanol was obtained from Spectrum chemicals, India. Methyl acrylate was procured from Loba chemicals, India. Ethylenediamine, ammonium molybdate, pyridine and diethyl ether were obtained from Merck, India. Ammonia and potassium iodide were obtained from SD fine chemicals, India. Rose Bengal was purchased from Aldrich, USA. Dialysis tubing (12-14 Kda cut-off size) was obtained from Himedia, India. All the chemicals were used as received except methanol which was distilled twice before use.

4.2. Characterization Techniques

Surface morphology of G2.5 PAMAM dendrimer was analyzed using atomic force microscopy (AFM) in contact mode using XE 70, SPM, Park system, South Korea, in a scan

area of 20 µm. The sample preparation was performed by taking 5 µL of G2.5 PAMAM and diluted by 100 times. The zeta potential analysis was performed at 25 °C, in MilliQ® water using Zetasizer nano, Malvern instruments, UK. A few drops of the prepared solution were allowed to spin coat on a glass substrate for ten minutes and then dried before measurement. UV-vis spectra (Lambda 25, Perkin Elmer, USA) of PAMAM dendrimers were measured in methanol: water (50% v/v). Fourier transform infra red (FTIR) spectra of the samples were recorded in liquid mode using a modern Bruker optic GmbH-Alpha T spectrometer, Germany.

4.3. Estimation of Drug Loading and Encapsulation Efficiency

The amount of drug loaded into the dendrimer and the encapsulation efficiency of the G2.5 PAMAM dendrimers were measured spectrophotometrically, using the formulae given below:

$$\text{Drug loading (\%)} = [W1/W2] \times 100 \quad (1)$$

$$\text{Encapsulation efficiency} = [(W_3 - W_4)/W_4] \times 100 \quad (2)$$

where W_1 = weight of the drug present in dendrimer, W_2 = net weight of the dendrimer, W_3 = weight of the drug added, W_4 = weight of the drug released into the supernatant.

4.4. Measurement of Drug Release Kinetics

The release of RB from the G2.5 PAMAM was measured spectrophotometrically as follows: 50 mg of RB encapsulated dendrimer was made up to 1 mL using a mixture of methanol: water (50:50, v/v).

This solution was dialyzed using a dialysis tubing with a MW cut-off 12000-14000 Da (~ 2.4 nm) (Himedia, India) against phosphate buffer saline of pH 7.4 at 37°C with mild stirring. This was continued for 72 hours and at each time interval 1 ml was withdrawn from the PBS for spectrophotometric analysis at λ_{max} 540 nm and was replaced by fresh PBS of same amount. A graph was plotted with cumulative release % against time interval in hours represents the drug release profile.

4.5. Light Source for PDT

A 150 W xenon arc lamp was used as a light source. The therapeutic window was adjusted by using 10% KI (5cms path length) and pyridine (1cm path length) as a filter for UV radiation. The specimen was kept in an open quartz cuvette and air saturated by magnetic stirring. It was irradiated at a distance of 12 cm from the light source.

4.6. Measurement of Quantum Yield of ROS Generation by Iodide Method

The iodide assay was used for the evaluation of ROS generation of RB encapsulated G2.5 PAMAM dendrimers (Mosinger et al. 1997). This assay is based on the reaction of singlet oxygen ($^1O_2$) (produced in the photodynamic reaction) with I^- in the presence of ammonium molybdate as a catalyst. The reaction product is I_3^- , the amount of which (measured spectrophotometrically at $\lambda = 351$ nm) is directly proportional to the generated $^1O_2$.

4.7. *In Vitro* Cell Viability Test - MTT Assay

For MTT cell viability assay, 2.5×10^4 DLA cells per well were seeded onto a well of 96-well plates in RPMI 1640 media for 2 h incubation, treated with various concentrations of RB in free and dendrimer encapsulated form and photo irradiated for 10 minutes using a xenon arc lamp. The media was changed to fresh RPMI 1640 media with 10 % PBS and incubated for 12 h. Then, 5 mg/mL MTT solution (20 µL/well) was added to each well, and cells were incubated for an additional 4 h at 37 °C. The supernatant was aspirated and 100 µL of isopropanol were added to the wells to dissolve any blue precipitate present. The absorbance was then measured at 570 nm by a micro plate reader. Cell viability was calculated using the following formula:

Cell viability = (Average absorbance of treated group/Average absorbance of the control group)×100 (3)

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